CDKN2BAS is associated with periodontitis in different European populations and is activated by bacterial infection

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ABSTRACT

Epidemiological studies have indicated a relationship between coronary heart disease (CHD) and periodontitis. Recently, CDKN2BAS was reported as a shared genetic risk factor of CHD and aggressive periodontitis (AgP), but the causative variant has remained unknown. To identify and validate risk variants in different European populations, we first explored 150 kb of the genetic region of CDKN2BAS including the adjacent genes CDKN2A and CDKN2B, covering 51 tagging single nucleotide polymorphisms (tagSNPs) in AgP and chronic periodontitis (CP) in individuals of Dutch origin (n=313). In a second step, we tested the significant SNP associations in an independent AgP and CP population of German origin (n=1264). For the tagSNPs rs1360590, rs3217992, and rs518394, we could validate the associations with AgP before and after adjustment for the covariates smoking, gender and diabetes, with SNP rs3217992 being the most significant (OR 1.48, 95% CI 1.19 to 1.85; p=0.0004). We further showed in vivo gene expression of CDKN2BAS, CDKN2A, CDKN2B, and CDK4 in healthy and inflamed gingival epithelium (GE) and connective tissue (CT), and detected a significantly higher expression of CDKN2BAS in healthy CT compared to GE (p=0.004). After 24 h of stimulation with Porphyromonas gingivalis in Streptococcus gordonii pre-treated gingival fibroblast (HGF) and cultured gingival epithelial cells (GECs), we observed a 25-fold and fourfold increase of CDKN2BAS gene expression in HGFs (p=0.003) and GECs (p=0.004), respectively. Considering the global importance of CDKN2BAS in the disease risk of CHD, this observation supports the theory of inflammatory components in the disease physiology of CHD.

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death worldwide[1][2] and is considered to be a complex multifactorial disease. Epidemiological studies have shown a significant correlation between periodontal health and the occurrence of cardiovascular events.[3][4] Periodontitis and CHD are both strongly promoted by very similar environmental and behavioural risk factors, with smoking and diabetes being the most prominent.[5–13] Furthermore, the same pathogenic bacterial strains were observed in both inflamed gingival tissue and atherosclerotic plaques.[10][14][15] It has also been shown that periodontal treatment results in

improvement of endothelial function, a marker for risk prediction of cardiovascular events.[16] Recently, a major genetic susceptibility locus for CHD was identified by various genome wide association studies (GWAS).[17–20] This locus is located within the chromosomal region 9p21.3, mapping to the large non-coding (nc) antisense RNA transcript CDKN2BAS, formerly called ANRIL. This association was verified by a subsequent meta-analysis[9] and by a large scale replication study,[21] making this genetic region the best replicated CHD associated risk locus to date. In addition, variants within this region were independently found to be associated with type 2 diabetes,[22–24] abdominal aortic and intracranial aneurysms,[25] ischaemic stroke,[26] Alzheimer's disease and vascular dementia,[27] and high grade glioma susceptibility.[28] Recently, we demonstrated the association of a highly increased risk for aggressive periodontitis (AgP) with specific variants of CDKN2BAS.[29] However, despite extensive fine mapping and functional studies, the causative variant and the functional role of CDKNBAS has remained unknown.[9][30] In recent years, long non-coding RNAs like CDKN2BAS have been recognised for their role in regulating gene expression by contributing to the recruitment of protein complexes to target genes in trans, and to provide an architectural matrix to retain the proteins at the repression site of the target gene. Unprecedented, a role in cis regulation of CDKN2A expression was very recently described for a new transcript of CDKN2BAS that initiates upstream of the previously reported start site,[31] indicating a regulatory role for CDKN2BAS in the context of cellular proliferation.

In the present study, we aimed to identify genetic risk variants of CDKN2BAS that are shared by different European case—control populations of aggressive and chronic periodontitis. To this end, we covered >150 kb with 51 tagging single nucleotide polymorphisms (tagSNPs) mapping to CDKN2BAS and the adjacent cell cycle inhibitor genes CDKN2A and CDKN2B. We explored the SNP associations in two populations of AgP and chronic periodontitis (CP) of Dutch origin (n=313), and replicated the significant associations in two populations of AgP and CP of German origin (n=1264).

Recently, differential gene expression of CDKN2BAS in a variety of CHD relevant tissues, such as vascular endothelial cells, smooth coronary

muscle cells, fibroblasts, and macrophages, has been demonstrated.[30] [32] [33] To study the tissue specific expression in the gingiva, we quantified the mRNA transcription pattern of *CDKN2BAS, CDKN2A* and *CDKN2B* in healthy and inflamed human gingival tissue samples. Additionally, we studied the gingival expression of *CDK4*, a cell cycle regulating kinase that is directly inhibited by CDKN2A and CDKN2B. To explore the possibility that both periodontitis and CHD share an inflammatory pathophysiology, we assessed the potential role of pathogenic bacteria in the transcriptional regulation of these genes in different gingival tissues.

PATIENTS AND METHODS
Study population
The patient and control samples of the CP and AgP association studies were recruited across Germany and the Netherlands as previously described.[34] Only individuals of German and Dutch ethnicity were included, judged upon the location of both parental birthplaces. Before the study, the genetic sub-structure of the German and Dutch populations had been assessed,[35] [36] indicating only negligible sub-structures and therefore allowing an analysis of the German and Dutch individuals in a single genetic study without the risk of substantial population stratification. A detailed description of the study population is given in table 1 and in the supplementary information. Informed written consent was obtained from all subjects recruited into this study. The study was approved by each institute's own ethical review board (Medical Ethical Committee, Universities of Bonn, Dresden, Kiel and Munich, and Medical Ethical Committee, Academic Medical Center, University of Amsterdam, The Netherlands).

DNA extraction and genotyping
Genomic DNA was extracted from blood (Invisorb Blood Universal Kit, Invitek, Berlin, Germany) or mouthwash samples,[37] and was amplified by whole genome amplification (GenomiPhi, Amersham, Uppsala, Sweden). TagSNPs were selected from the CEU data of the International HapMap project (http://www.hapmap.org, NCBI build 36, 2008-03) using the Tagger feature[38] of Haploview 4.0.[39] TagSNPs were defined by pair-wise linkage disequilibrium tagging ($r^2 > 0.8$). Genotyping was carried out using the SNPlex and TaqMan Genotyping System (Applied Biosystems, Foster City, California, USA) on an automated platform, using TECAN Freedom EVO and 96-well and 384-well TEMO liquid handling robots (TECAN, Männedorf, Switzerland). Genotypes were generated by automatic calling using the Genemapper 4.0 software (Applied Biosystems) with the following settings: ς separation >6, angle separation for two cluster SNPs <1.2 rad, median cluster intensity >2.2 logs. Genotypes were further reviewed manually and call rates ≥90% in each sample set were required.

Gingival tissue samples and cell culture
For gene expression study, gingival tissue samples were obtained from individuals with healthy gingival conditions and no periodontitis (n=10) as well as from patients with advanced CP (n=10). All biopsies were collected from healthy patients (age 19—58 years) who underwent third-molar extraction or periodontal surgery and from patients with advanced CP. Healthy gingival conditions were defined as follows: no redness, no swelling, no bleeding on probing, and with periodontal pockets ≤3 mm. Patients with advanced CP showed all typical clinical signs of periodontal inflammation (redness, swelling, bleeding on probing) and a periodontal probing depth ≥6 mm. For culture of gingival epithelial cells (GECs) (n=3) and human gingival fibroblasts (HGFs) (n=3), gingival epithelial tissue samples were prepared as previously described.[40] Epithelial cells were cultured in keratinocyte growth medium (KGM) using supplements provided with the medium (PAA, Cölbe, Germany) and 1% antibiotics (penicillin, streptomycin, amphotericin; PAA), whereas fibroblasts were cultured using Dulbecco's modified Eagle's medium (DMEM; PAA) supplemented with 1% antibiotics (penicillin, streptomycin, amphotericin; PAA) and 10% fetal bovine serum (FBS; PAA). Both GECs and HGFs were cultured at 37°C in a humidified atmosphere (5% carbon dioxide).

Bacterial cell culture and stimulation experiments
Wild-type *Porphyromonas gingivalis* strain ATCC 33277 was cultured under anaerobic conditions, whereas *Streptococcus gordonii* was grown under aerobic conditions as previously described.[41] [42] Bacterial numbers were determined by absorbance measurement using a spectrophotometer (Biochrom AG, Berlin, Germany). To mimic early stages of inflammation and to test

Table 1 Characteristics of the study populations

	Dutch			German		
	Aggressive periodontitis	Chronic periodontitis	Controls	Aggressive periodontitis	Chronic periodontitis	Controls
Subject characteristic						
Individuals, N (%)	159	154	421	301	740	962
Men	41 (25.8)	56 (36.4)	185 (43.9) 5 ns	112 (37.2)	302 (40.8), 5 ns	559 (58.1), 1 ns
Women	118 (74.2)	98 (63.6)	231 (54.9)	189 (62.8)	433 (58.5)	402 (41.8)
Mean age (SD) at first diagnosis	30.4 (4.2)	ns	—	29.5 (4.7)	ns	—
Mean age (SD) at examination	33.8 (4.7)	45.2 (9.5)	32.3 (9.0)	31.4 (6.1)	61.2 (10.3)	56.9 (12.7)
Study information						
Recruitment period	2003—2008	1995—2004	1995—2008	2003—2008	2000—2006	2002—2006
Phenotype						
Affected teeth, mean (SD)	5.8 (3.9)	ns	—	8.6 (6.4)	ns	—
Risk factor, N (%)						
Ever smoked	123 (77.4)	128 (83.1) 1 ns	197 (46.8) 0 ns	147 (48.8)	487 (65.8) 6 ns	492 (51.1) 4 ns
Diabetes mellitus	1 (0.6) 5 ns	1 (0.6) 1 ns	2 (0.2) 354	3 (1.0) 67 ns	37 (5.0) 5 ns	44 (4.6) 3 ns

Values are given as mean (SD) when appropriate. Affection status was based on reported history. Age was reported by the clinicians. Smoking was estimated by self-report of the individuals. ns, not specified.

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the potential transcriptional response of these genes, the commensal oral bacterium *S gordonii* and the pathogenic oral bacterium *P gingivalis*, commonly present during severe periodontal infection, were chosen to create an in vitro model to mimic periodontal infection of GECs and HGFs. For stimulation experiments, GECs and HGFs were grown to 80% confluence and then incubated with *P gingivalis* and *S gordonii*, respectively, using an amount equivalent to a multiplicity of infection of 100:1, for either 6 h or 24 h. Experimental groups were: (1) unstimlated GECs and HGFs; (2) control cells (GECs and HGFs) treated with blank bacterial medium for *S gordonii* not containing bacterial cells; (3) control cells (GECs and HGFs) treated with blank bacterial medium for *P gingivalis* not containing bacterial cells; (4) stimulation of GECs and HGFs with *S gordonii*; (5) stimulation of GECs and HGFs with *P gingivalis*; and (6) pre-incubation of GECs and HGFs with *S gordonii* followed by medium change and stimulation with *P gingivalis*. Each stimulation experiment was performed in triplicate, using cells from three different donors.

RNA extraction and PCR conditions

To extract mRNA from the epithelial and the connective tissue layer separately, biopsies were enzymatically dissected using 3 ml dispase (Cascade Biologics, Paisley, UK) for 6 h on ice. After separation of gingival biopsies, total RNA was extracted from both epithelium and connective tissue (CT) using the RNeasy Protection Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Subsequent to in vitro stimulation experiments on GECs and HGFs, total RNA was extracted as described above. The reverse transcription reaction with 500 ng of total RNA was performed using iScript (Bio-Rad, Munich, Germany) following the manufacturer's instructions.

Quantitative analysis of the cDNA was performed using the iCycler (Bio-Rad) and the QuantiFast SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. PCR reactions were carried out in 96-well plates in a total volume of 25 µl, including 10 ng of cDNA and 250 nM primers. At the end of each real-time PCR, melting curve analysis was performed to confirm that the amplified product was specific. Gene expression levels in the gingival epithelium and the gingival connective tissue were analysed by normalisation to the gene expression levels in healthy gingival epithelium. All sample values were normalised to the expression of the housekeeping gene glycerylaldehyde-3-dehydrogenase (GAPDH, GeneGlobe primer, Qiagen) and relative gene expression was calculated using the delta-delta Ct (threshold cycle) method. PCR controls were performed using water. Primer sequences are given in supplementary table 1.

Statistical analysis

Markers were checked for deviation from Hardy—Weinberg equilibrium (HWE) in controls to a significance level of $\alpha = 0.05$ before inclusion into the analysis. We assessed the significance of phenotypic association with single marker alleles and genotypes using χ^2 and Fisher's exact tests for 2×2 and 2×3 contingency tables, respectively. Logistic regression analysis allowing for covariate adjustment was performed in the R statistical environment, version 2.8.1.[43] Significance was assessed by a likelihood ratio test. Values of $p \leq 0.05$ were considered nominally significant. Akaike's information criterion (AIC) was used to choose the model that best explained the underlying associations. Linkage disequilibrium (LD) measures were calculated and plotted using Haploview. Quantitative real time (qRT)-PCR

(qRT-PCR) data were analysed using the Mann—Whitney Test (SPSS, Version 18, Munich, Germany). The significance level was set at $p \leq 0.05$.

RESULTS
Association study with aggressive periodontitis

To analyse potential genetic associations within the entire *CDKN2BAS* region, we selected 51 tagSNPs covering 150 kb. These SNPs were genotyped in our combined population of 159 Dutch generalised and localised AgP patients and 421 Dutch healthy controls. In our previous study on the association of *CDKN2BAS* with AgP,[29] we demonstrated that the allele frequencies at this genetic locus were very similar between localised and generalised AgP cases, allowing the pooling of both sub-phenotypes to increase the statistical power for detecting associated variants. In a second step, we validated the significant SNPs in an independent panel of our 301 German patients with generalised or localised AgP and 963 German controls.[29] In a third step, we subsequently tested the selected tagSNPs in this genetic region for potential associations with chronic periodontitis in a panel of 154 Dutch CP cases, and replicated the significant associations in an independent panel of 740 German CP cases. To increase the statistical power, we used the same panels of Dutch and German controls for the association tests performed with the Dutch and German AgP and CP patients, respectively (table 1).

Out of the 51 tagSNPs, eight were found to have a minor allele frequency (MAF) below 0.01 in the Dutch populations. These were excluded from the analysis due to their limited expected statistical power. Five further SNPs were excluded due to deviations from HWE in the Dutch controls (p<0.05). One SNP, rs564398, had poor genotyping quality (call rate <0.9) and was excluded from further analyses. In the explorative association study of the Dutch AgP case—control population, four SNPs were nominally significantly associated with AgP before adjustment for the common risk factors of gender, smoking, and diabetes (supplementary table 2). Upon covariate adjustment, seven SNPs were nominally significantly associated (table 2, for genotype and allele frequencies see table 3), with SNP rs3217992 being the most significant (OR 2.53, 95% CI 1.29 to 5.13; p=0.007). Two SNPs, rs1360590 and rs11790231, showed associations before and after covariate adjustment (supplementary tables 2 and 3).

These seven nominally significantly associated SNPs were then tested for their association in the German AgP population. One SNP, rs10965224, showed significant deviations from HWE in the German controls (p<0.05) and was excluded from further analyses. Before the covariate adjustment, three of the associations remained significant (rs3217992, rs518394, rs1360590), with SNP rs1360590 being most significantly associated with p=0.0005 (multiplicative genetic model) and an OR of 1.41 (CI 95% 1.16 to 1.72) (supplementary table 4). Upon covariate adjustment, these three SNPs remained significantly associated, with rs3217992 being most significant with p=0.0004 (table 2, supplementary table 5) and an OR of 1.48 (95% CI 1.19 to 1.85). After Bonferroni correction for multiple testing, these three SNPs remained significant at the corrected significance threshold of $p \leq 0.0083$ for six independent tests. These three SNPs span a chromosomal region of 38 kb (NCBI build 36.3) and are located within (rs3217992) or downstream of *CDKN2B*, but were only in moderate LD to each other in our control population (figure 1). Interestingly, none of the SNPs which tagged the large LD region at the 5′ end of *CDKN2BAS*, and which was most significantly associated with CHD [9] [17–19] [20] as well as with AgP

Table 2 Minor allele frequencies (MAFs) and single nucleotide polymorphism (SNP) associations of the significant SNPs in the different populations upon adjustment for covariates

SNP (alleles)	Dutch controls	German controls	Dutch AgP		German AgP			Dutch CP			German CP			
	MAF	MAF	p	OR (CI 95%)	MAF	p	OR (CI 95%)	MAF	p	OR (CI 95%)	MAF	p	OR (CI 95%)	MAF
rs3217992 (G,A)	37.1	35.0	7.2×10^{-3}	2.53 (1.3 to 5.1)	34.0	4.4×10^{-4}	1.48 (1.2 to 1.9)	43.3	0.060	1.82 (1.0 to 3.4)	38.9	NA	NA	NA
rs518394 (C,G)	44.5	46.1	0.045	2.32 (1.1 to 4.9)	50.3	7.8×10^{-3}	0.74 (0.6 to 0.9)	39.3	0.114	0.56 (0.3–1.2)	39.2	NA	NA	NA
rs1360590 (A,G)	47.5	44.2	0.012	2.6 (1.3 to 5.2)	42.8	2.0×10^{-3}	1.41 (1.1 to 1.8)	52.9	0.029	2.1 (1.1 to 3.9)	50.0	0.119	1.22 (1.0 to 1.6)	46.4
rs11790231 (G,A)	11.4	9.0	0.039	NA	9.1	0.08	1.35 (1.0 to 1.8)	10.7	0.504	0.5 (0.1 to 4.4)	8.9	NA	NA	NA
rs10965238 (A,G)	28.5	24.2	0.024	0.30 (0.1 to 0.9)	24.9	0.367	1.12 (0.9 to 1.4)	25.4	3.0×10^{-3}	0.48 (0.3 to 0.8)	20.2	0.259	0.89 (0.7 to 1.1)	23.6
rs10811658 (G,A)	33.8	30.8	0.047	0.40 (0.16 to 0.99)	29.7	0.055	1.2 (1.0 to 1.5)	34.0	0.024	0.59 (0.4 to 0.9)	26.2	0.025	0.89 (0.7 to 1.1)	28.3

Given are the MAFs, the p values, and the ORs with the 95% CIs for the different populations. The p values were obtained from a likelihood ratio test and correspond to the genetic model with the lowest AIC value each. Values are given upon adjustment for the covariates smoking, diabetes, and gender for the aggressive periodontitis (AgP) analyses and additionally for age for the chronic periodontitis (CP) analyses, in a logistic regression model.

in our German cases,[29] was significantly associated with the Dutch AgP patients in this study. The lead SNPs of this region were associated with CHD and the German AgP cases with a 10% increase of the rare allele in the homozygotes (eg for SNP rs1333048) and showed similar allele frequencies for the heterozygotes between cases and controls. In contrast, for the Dutch AgP cases we observed an enrichment of 10% in the heterozygotes but a decrease of 6% of the rare allele in the homozygotes (supplementary table 6).

Association study with chronic periodontitis

In the next step, we tested the 37 tagSNPs that had passed the pre-assigned QC for their association with the phenotype chronic periodontitis in the explorative Dutch CP population. Five SNPs were significant before covariate adjustment for gender, age, smoking, and diabetes, with SNP rs10965238 being the most significant (OR 0.57, 95% CI 0.38 to 0.84; p=0.004) (supplementary table 7). Upon adjustment, four SNPs remained significant (rs1360590, rs10965224, rs10965238, rs10811658), two of which showed stable associations before and upon covariate adjustment (rs10965238, rs10811658) (figure 1, table 2 and supplementary table 8).

We next tested these three SNPs for their association in the German CP population, again excluding SNP rs10965224 due to its significant deviations from HWE in this control population. Before covariate adjustment, SNP rs10811658 was significantly associated with CP (supplementary table 9). Upon adjustment, this association remained significant in a similar fashion with p=0.025 (OR 0.66, 95% CI 0.46 to 0.95) (table 2). However, after Bonferroni correction for multiple testing, this association

missed significance at the corrected significant threshold of p<0.0167 for three independent tests. None of the other SNPs tested in this case—control population showed significant associations upon covariate adjustment (supplementary table 10).

CDKN2BAS, CDKN2A, CDKN2B, and CDK4 mRNA expression in healthy and inflamed gingival tissues

To show in vivo gene expression of CDKN2BAS, CDKN2A, CDKN2B, and CDK4, the specific mRNA levels of these genes within the epithelium (EPI) and the connective tissue (CT) of gingival biopsies were analysed. We observed tissue specific expression of all four genes in both EPI and CT. In this explorative study, the expression of CDKN2BAS was significantly higher in healthy CT compared to healthy EPI (p=0.004, figure 2A), and this difference remained significant after Bonferroni correction for multiple comparisons. Whereas for CDKN2A no difference in transcript levels was observed between CT and EPI (figure 2B), higher mRNA levels, although not significant in our experiments, were observed for CDKN2B in healthy CT compared to healthy EPI (p=0.019, figure 2C). A trend towards a lower gene expression for CDK4, inhibited by CDKN2A and 2B in vivo, was noticed in CT compared to EPI (figure 2D).

CDKN2BAS gene expression upregulated in S gordonii pre-treated and P gingivalis stimulated GECs

We next tested whether the genes were differentially regulated in cultured primary gingival epithelial cells (GECs) when exposed to commensal and/or pathogenic periodontal bacteria. We observed a significant increase of the CDKN2BAS gene expression after 24 h of stimulation with pathogenic P gingivalis

Table 3 Genotypes and frequencies of the significant single nucleotide polymorphisms (SNPs) in the different populations

| SNP (alleles)	Dutch controls			German controls			Dutch aggressive periodontitis (AgP)			German AgP			Dutch chronic periodontitis (CP)			German CP		
	11(%)	12(%)	22(%)	11(%)	12(%)	22(%)	11(%)	12(%)	22(%)	11(%)	12(%)	22(%)	11(%)	12(%)	22(%)	11(%)	12(%)	22(%)
rs3217992 (G,A)	171 (41.1)	182 (43.6)	64 (15.3)	281 (42.5)	298 (45.0)	83 (12.5)	66 (41.5)	78 (49.1)	15 (9.4)	96 (32.3)	145 (48.8)	56 (18.9)	60 (39.2)	67 (43.8)	26 (17.0)	NA	NA	NA
rs518394 (C,G)	132 (31.5)	201 (48.0)	86 (20.5)	197 (29.7)	321 (48.4)	145 (21.9)	38 (23.9)	82 (51.6)	39 (24.5)	113 (37.7)	138 (46.0)	49 (16.3)	60 (39.3)	66 (43.1)	27 (17.6)	NA	NA	NA
rs1360590 (A,G)	122 (29.0)	198 (47.0)	101 (24.0)	207 (31.2)	327 (49.2)	130 (19.6)	48 (30.2)	86 (54.1)	25 (15.7)	64 (21.4)	154 (51.5)	81 (27.1)	41 (26.6)	72 (46.8)	41 (26.6)	219 (29.7)	352 (47.8)	166 (22.5)
rs11790231 (G,A)	330 (78.6)	84 (20.0)	6 (1.4)	551 (83.2)	104 (15.7)	7 (1.1)	130 (81.8)	29 (18.2)	0 (0.0)	238 (79.9)	56 (18.8)	4 (1.3)	129 (84.3)	21 (13.7)	3 (2.0)	NA	NA	NA
rs10965238 (A,G)	202 (51.4)	158 (40.2)	33 (8.4)	363 (58.4)	216 (34.8)	42 (6.8)	86 (56.2)	58 (37.9)	9 (5.9)	160 (56.2)	105 (36.8)	20 (7.0)	97 (65.1)	44 (29.5)	8 (5.4)	420 (59.9)	232 (33.0)	50 (7.1)
rs10811658 (G,A)	181 (43.2)	193 (46.1)	45 (10.7)	324 (49.1)	266 (40.3)	70 (10.6)	77 (48.8)	68 (43.0)	13 (8.2)	130 (43.4)	135 (45.2)	34 (11.4)	84 (54.9)	58 (37.9)	11 (7.2)	374 (50.7)	310 (42.1)	53 (7.2)
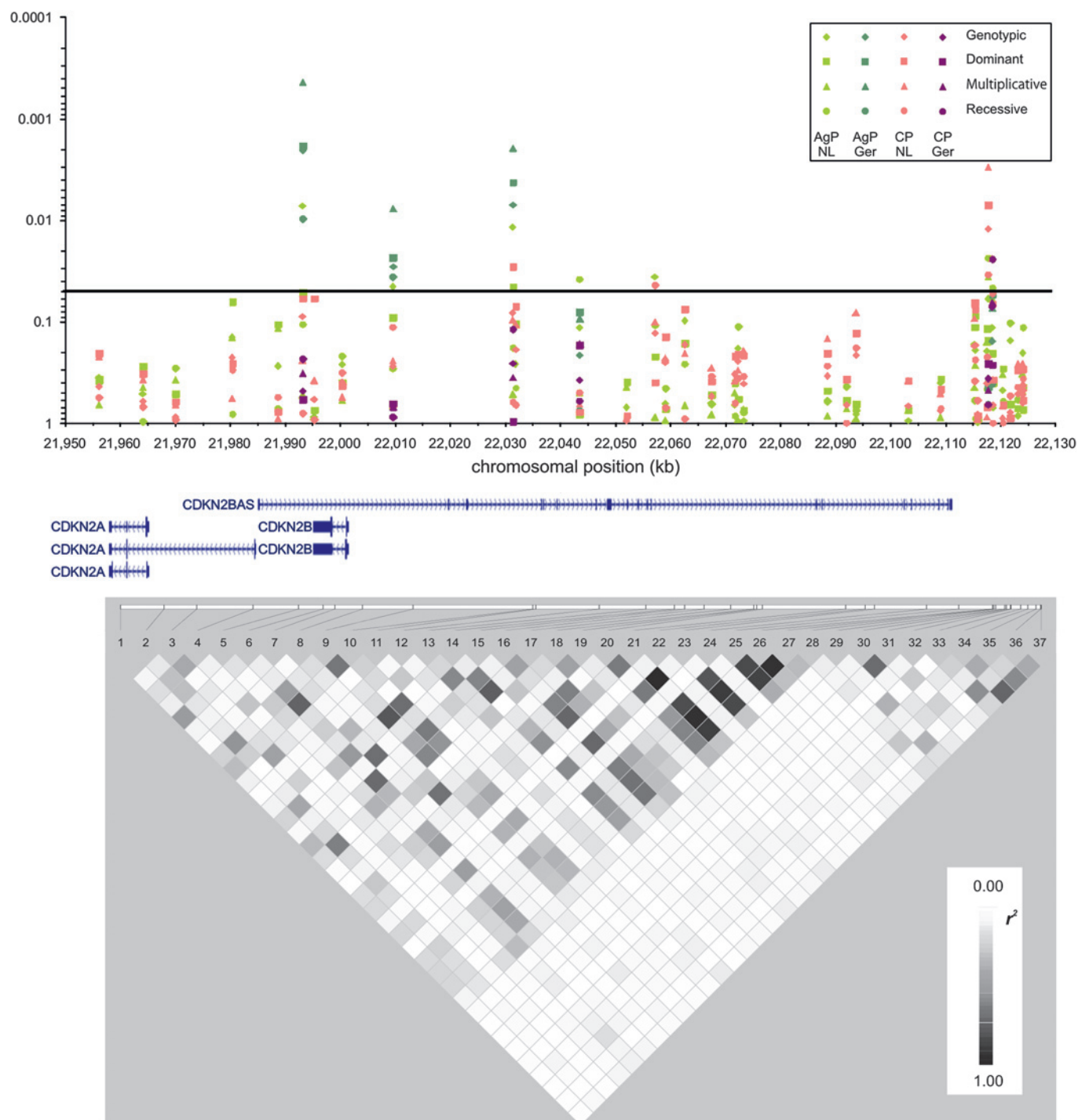
Figure 1   Genetic region of *CDKN2BAS*, The top diagram shows the nominal $-\log_{10}$ p under the assumption of the different genetic models of those single nucleotide polymorphisms (SNPs) that passed the preassigned quality criteria. The $-\log_{10}$ p values are plotted as a function of the genomic htSNP position (NCBI build 36). The bottom panel shows pairwise linkage disequilibrium (LD) in the controls using metric r^2. AgP, aggressive periodontitis; CP, chronic periodontitis; Dominant, dominant model; Genotypic, genotypic model; Ger, Germany; NL, Netherlands; Recessive, recessive model; bp, base pairs; 1, rs3731257; 2, rs3731239; 3, rs2811709; 4, rs7036656; 5, rs3218009; 6, rs3217992; 7, rs3217986; 8, rs495490; 9, rs518394; 10, rs1360590; 11, rs17694493; 12, rs11790231; 13, rs1011970; 14, rs10965224; 15, rs16905599; 16, rs10757270; 17, rs1412832; 18, rs10965227; 19, rs10965228; 20, rs1333040; 21, rs2891168; 22, rs10125231; 23, rs1333042; 24, rs13301964; 25, rs1333045; 26, rs1333048; 27, rs1333049; 28, rs1333050; 29, rs10757281; 30, rs10965238; 31, rs10811658; 32, rs12347779; 33, rs10965245; 34, rs2891169; 35, rs7045889; 36, rs10811661; 37, rs10757283.

in commensal *S gordonii* pre-treated GECs with p=0.003 (four-fold increase), and this difference remained significant after Bonferroni correction for multiple comparisons (figure 3A). In the same experiments, *CDKN2A* mRNA levels were significantly reduced after 24 h of stimulation with pathogenic *P gingivalis* in

commensal *S gordonii* pre-treated GECs with p=0.004, if compared to unstimulated GECs (figure 3B). A similar reduction, not significant in our experiments, was observed between 6 h and 24 h of *P gingivalis* stimulation after *S gordonii* pre-treatment. For *CDKN2B*, we observed a trend towards reduced

Figure 2 mRNA levels of *CDKN2BAS*, *CDKN2A*, *CDKN2B* and *CDK4* in healthy and inflamed gingival epithelium and connective tissue. (A) *CDKN2BAS* transcript levels were higher in healthy connective tissue compared to healthy gingival epithelium (p=0.004). (B—D) *CDKN2A*, *CDKN2B*, and *CDK4* mRNA showed no statistically significant differences in transcript levels between healthy or inflamed epithelial and connective gingival tissues. Each box plot represents results derived from 10 different subjects. CT, connective tissue; EPI, gingival epithelial tissue; H, healthy; I, inflamed.
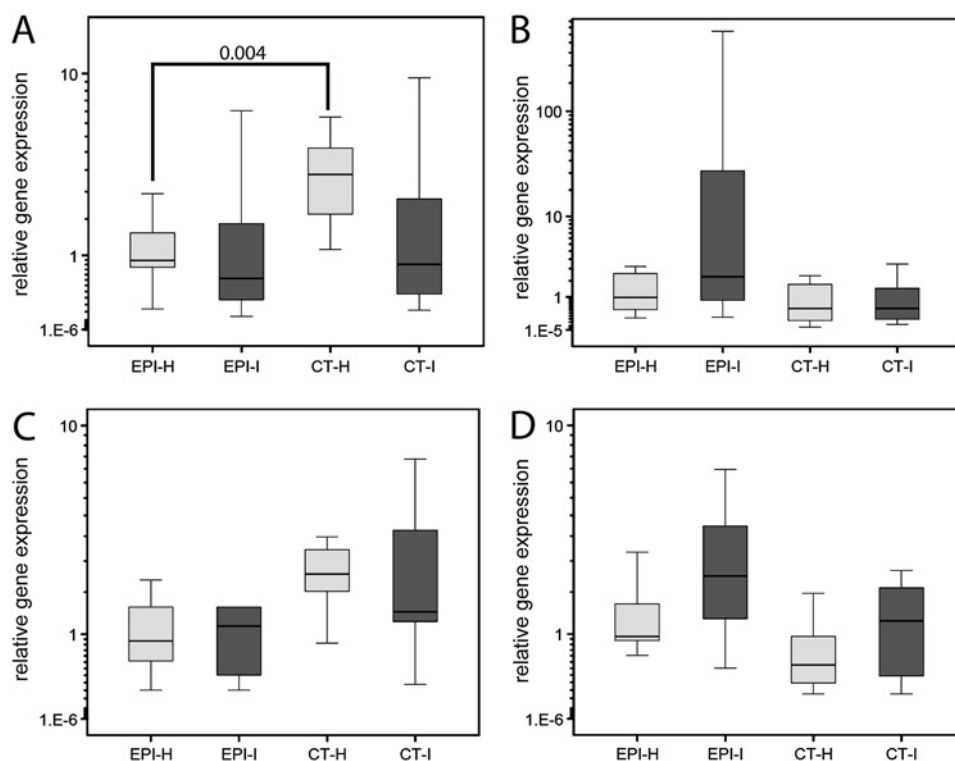
transcript levels after 24 h incubation with *P gingivalis* in *S gordonii* pre-treated GECs (figure 3C). *CDK4* expression was significantly reduced after 24 h of stimulation with pathogenic *P gingivalis* in commensal *S gordonii* pre-treated GECs compared to unstimulted GECs (p=0.004, figure 3D). A similar reduction, not significant in our experiments, was observed between 6 h and 24 h *P gingivalis* stimulation after *S gordonii* pre-treatment.

CDKN2BAS gene expression upregulated in *P gingivalis* stimulated HGFs

In the second gingival cell population of gingival fibroblasts (HGFs), 24 h stimulation with *P gingivalis* alone and after pre-treatment with *S gordonii* led to significant *CDKN2BAS* mRNA upregulation after 24 h with p=0.004 and p=0.003 (25-fold), respectively, and remained significant after Bonferroni correction for multiple comparisons (figure 4A). *CDKN2A* mRNA levels were significantly reduced (p=0.001) after 24 h stimulation with *P gingivalis* after pre-treatment with *S gordonii* (figure 4B). No clear trend in transcript levels could be observed for *CDKN2B* and *CDK4* in any experimental condition (figure 4C,D).

DISCUSSION

The aim of this study was to identify genetic risk alleles within the genetic region of *CDKN2BAS*, *CDKN2A* and *CDKN2B*, which are shared by different European populations of the periodontal disease phenotypes AgP and CP. We showed three SNPs to be significantly associated with two AgP populations of different ancestry (rs3217992, rs518394, and rs1360590), and confirmed these associations to be independent of the traditional risk factors of smoking, diabetes and gender. These SNPs were located within or closely downstream of the coding region of *CDKN2B*, but no SNP could be identified as a lead SNP that fully explained the associations with periodontitis.

Interestingly, we could not replicate our previous finding of a statistically significant association of the large CHD associated LD region at the 5' end of *CDKN2BAS* with the Dutch AgP patients. As described earlier,[29] in the German AgP cases the frequency of the rare allele of the lead SNP from this study, rs1333048, was 10% higher compared to the controls and the frequencies for the heterozygotes was similar to the controls. This observation was similarly reported by association studies on CHD.[9] Interestingly, in the present study, the Dutch AgP cases showed an enrichment of 10% in the heterozygotes for this SNP, but the homozygotes of the risk allele were decreased. We ruled out potential genotyping errors of this assay by verifying the genotypes of SNP rs1333048 with two independent technologies, which gave identical results. Increased heterozygotes in the Dutch AgP cases were similarly observed for the significantly associated SNPs rs1360590, rs3217992, and rs518394. In contrast to the observation in the Dutch AgP patients, the Dutch CP cases, and to a lesser extent the German CP cases, showed a tendency towards an increased frequency of homozygosity for the risk allele, with a frequency of heterozygotes similar to the controls. A possible speculative explanation for the observed increase of heterozygous Dutch AgP patients at this particular locus could be the presence of duplicated structural variations of this element in these cases, which were not present in the Dutch controls and CP cases.

The discovered associations, which were shared between different populations, did not follow a particular model of inheritance (genotypic, dominant, recessive or multiplicative). However, this observation is rather typical for association studies of our sample sizes, where it is practically impossible to assess confidently which model fits the data best, and where the specific models might reflect noise rather than a specific fit to a model. A future meta-analysis with data across many different datasets will improve this situation and might help to identify the best-fit model.[44] The genetic effects that were estimated for the associated SNPs also partly differed between the populations. Corresponding to the variations in the genetic best-fit models, the genetic effects are statistical estimates which are liable to estimation errors, which are mostly attributed to effects of individual random samples which constitute

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Figure 3 mRNA levels of *CDKN2BAS*, *CDKN2A*, *CDKN2B*, and *CDK4* in gingival epidermal cells after stimulation with *Streptococcus gordonii* and *Porphyromonas gingivalis*. (A) After 24 h of bacterial infection, *CDKN2BAS* mRNA expression was significantly increased upon 24 h *P gingivalis* stimulation of *S gordonii* pre-treated gingival epithelial cells (GECs) compared to 6 h of stimulation (p=0.003). (B) *CDKN2A* gene expression was significantly reduced in response to 24 h of *P gingivalis* stimulation after *S gordonii* pre-treatment compared to unstimulated GECs (p=0.004). A similar reduction, not significant in our experiments, was observed between 6 h and 24 h of *P gingivalis* stimulation after *S gordonii* pre-treatment. (C) For *CDKN2B*, no significant differences in mRNA transcript levels were observed in GECs upon bacterial stimulation. (D) *CDK4* mRNA levels were significantly reduced after *S gordonii* pre-treated followed by 24 h of stimulation with *P gingivalis* compared to unstimulated GECs (p=0.004). A similar reduction, not significant in our experiments, was observed between 6 h and 24 h of *P gingivalis* stimulation after *S gordonii* pre-treatment. Each box plot represents values derived from nine independent experiments and cells from three different donors were tested. C, unstimulated cells; GEC, gingival epidermal cells; MPG, blank medium for *P gingivalis*; MSG, blank medium for *S gordonii*; PG, *P gingivalis*; SG, *S gordonii*; SG-PG, pre-treatment with *S gordonii* for 1 h followed by *P gingivalis* stimulation for 6 h and 24 h.
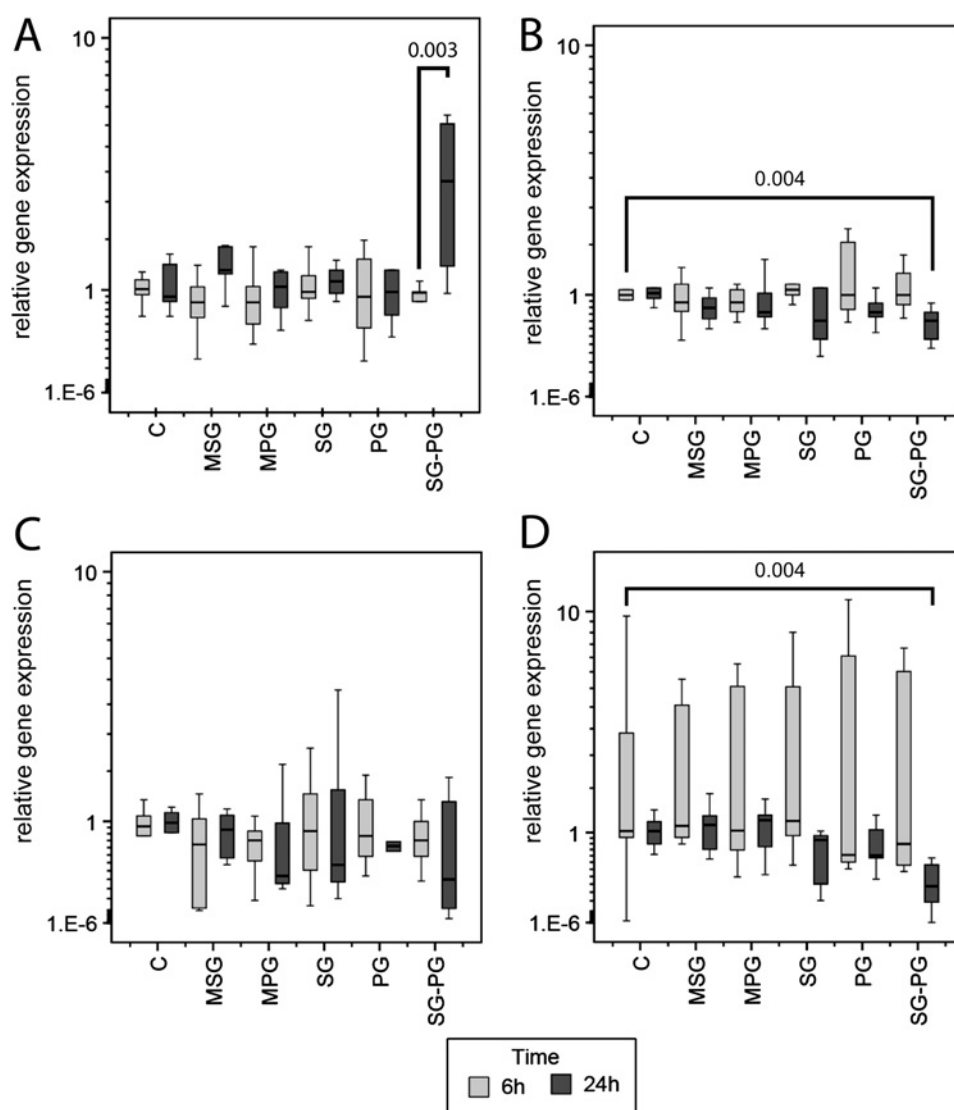
each population analysed. If the causative variant has not been identified, the observed SNP associations reflect the genetic effect of the causative variant through shared LD, which can vary between populations of different ancestry.

Although this broad validation study of the previously reported association of *CDKN2BAS* with periodontitis adds further evidence that variants at this locus increase the risk of periodontitis in individuals of Northern European origin, it is evident from the number and distribution of the significant SNP associations—which were observed in our current and our previous study, and similarly in other larger studies on CHD[9 33]—that the causative variant(s) and the related disease mechanisms remain to be identified. Additionally, we observed nominally significant SNP associations in the CP populations. Considering that individuals who experienced early development of periodontitis are likely to have inherited a major genetic disease variant, it is conceivable that in larger populations of the rather moderate phenotype chronic periodontitis, these SNPs may show their significance for CP as well as they did for the more severe AgP phenotype. To clarify this, replication experiments in further large populations of CP would be desirable.

The risk of periodontitis related to this locus may vary among different ethnic groups, which is suggested by frequency variations of the disease associated rs1333048-C allele from 21% in Yoruba to 51% in European and Japanese in HapMap. To identify the true causative variant(s), that may also be different between various ethnic groups, further genetic exploration of this genetic region in much larger analysis populations, including large analysis populations of various ethnic backgrounds, is necessary. A technical limitation of our study was the failure to genotype 16 of the selected tagSNPs in all populations, especially when the moderate LD across most of the genetic region of *CDKN2BAS* is considered. Here, an LD region harbouring the potential true variant(s) might have been missed. Future studies that will apply deep resequencing of cases carrying the associated variants across *CDKN2BAS* will obtain the full spectrum of variation in this region, and will be able to identify the causal variant(s) that affect the genetic risk.

In the second part of our study, we analysed the expression patterns of *CDKN2BAS*, *CDKN2A*, *CDKN2B*, and *CDK4* in healthy and inflamed gingiva, and assessed if these genes were regulated by a signal transduction cascade sensitive to bacterial infection. All analysed genes were expressed in the gingiva, with *CDKN2BAS* and *CDKN2B* both showing the highest mRNA levels of the analysed genes in CT. We did not observe different transcript levels between healthy or inflamed gingival tissues. This could indicate that these genes are not regulated by the

Figure 4 mRNA levels of *CDKN2BAS*, *CDKN2A*, *CDKN2B* and *CDK4* in human gingival fibroblasts after stimulation with *Streptococcus gordonii* and *Porphyromonas gingivalis*. (A) *CDKN2BAS* mRNA levels were significantly increased in non-*S gordonii* pre-treated (p=0.004) and *S gordonii* pre-treated human gingival fibroblasts (HGFs) (p=0.003) after 24 h of stimulation with *P gingivalis*. (B) *CDKN2A* mRNA levels were significantly reduced in *S gordonii* pre-treated and 24 h *P gingivalis* stimulated HGFs compared to unstimulated HGFs (p<0.001). (C, D) No significant differences of mRNA levels were observed for *CDKN2B* and *CDK4* mRNA levels in either condition. Each box plot represents values derived from nine independent experiments and cells from three different donors were tested. C, unstimulated cells; HGF, human gingival fibroblasts; MPG, blank medium for *P gingivalis*; MSG, blank medium for *S gordonii*; PG, *P gingivalis*; SG, *S gordonii*; SG-PG, pre-treatment with *S gordonii* for 1 h followed by *P gingivalis* stimulation for 6 h and 24 h.
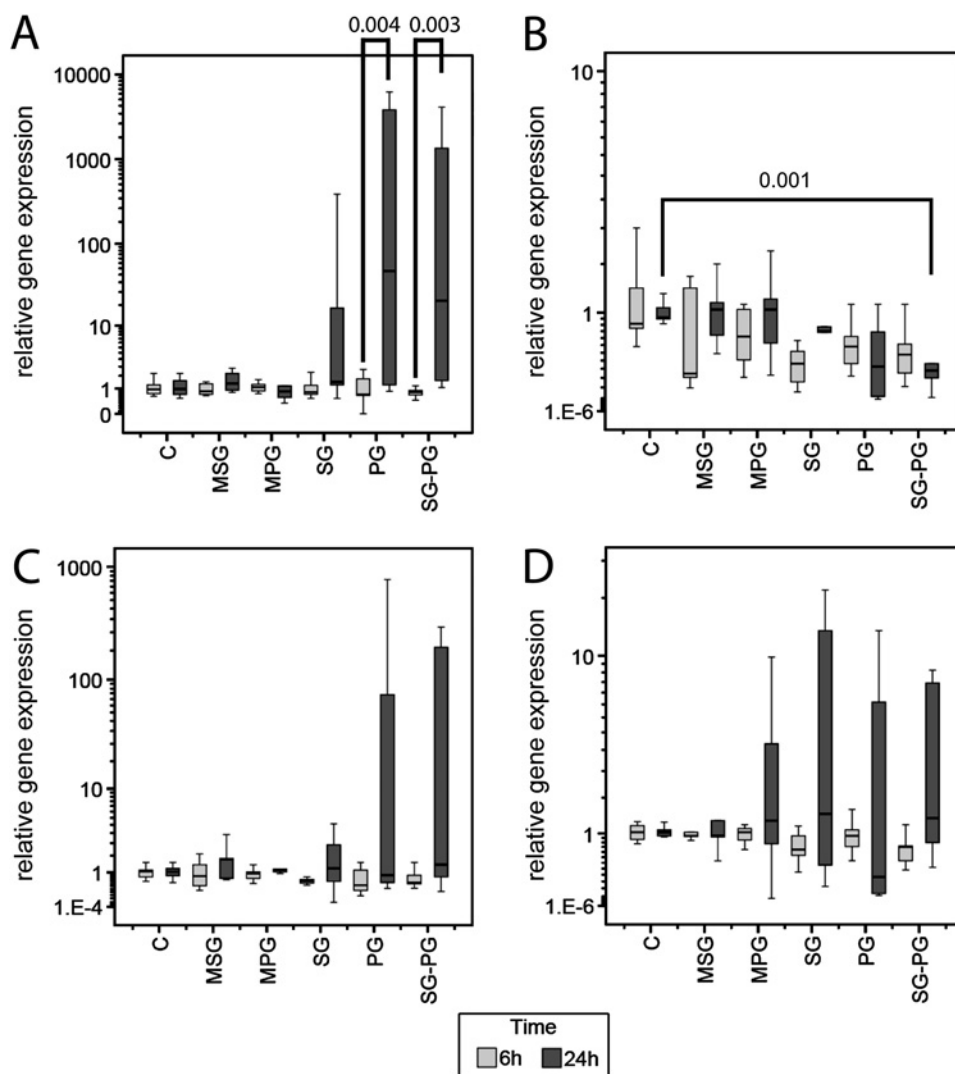
inflammatory status of the gingiva. However, although healthy gingival samples were taken from areas with no clinical signs of inflammation, surfaces of the oral cavity are constantly exposed to both commensal and pathogenic microorganisms. Therefore, it can be speculated that the clinically healthy gingiva is subjected to challenges similar to those of the early stages of infection. Likewise, gingival samples with periodontitis represent a tissue where constant infection and chronic inflammation occurred over years, and short term responses on the transcriptional level are most likely missed in inflamed gingival tissue samples. Here, an experimental setting that precisely controls for pathogenic infection might be a better way to assess specific transcriptional responses.

Because the functional variant(s) are as yet unknown for both periodontitis as well as for CHD, we did not aim to study the putative biological effects of the associated variants. Thus, we did not distinguish between the allelic background in our cell cultures, but studied the transcription pattern of the selected genes in response to bacterial infection. We observed that after 24 h of stimulation of healthy HGFs and GECs with the pathogenic bacterium *P gingivalis*, *CDKN2BAS* was significantly upregulated compared to the unstimulated controls. This real-time response of *CDKN2BAS* gene activity to a specific pathogenic inflammatory trigger indicates that *CDKN2BAS* is subjected to pathogen sensitive transcriptional regulation. Furthermore, the clear response after 24 h of stimulation, but

the absence of elevated transcript levels after 6 h of stimulation, suggests that *CDKN2BAS* may be located at a downstream position of a putative pathogen sensitive signalling cascade. We further observed a minor but significant downregulation of *CDKN2A* after 24 h of bacterial stimulation in both gingival epidermal tissue and gingival fibroblasts. These large quantitative differences in *CDKN2BAS* and *CDKN2A* transcript levels in response to 24 h bacterial stimulation possibly indicate that *CDKN2A*, unlike *CDKN2BAS*, is not directly regulated by bacterial infection on the transcriptional level, but at a later stage of this signal transduction cascade.

Our observations were in accordance with a very recent study on *CDKN2BAS* function which showed that *CDKN2BAS* transcripts negatively regulate *CDKN2A* expression.[31] This recent study detected that *CDKN2BAS* transcript initiates in prostate cancer tissues at a start site several hundred bases upstream of the previously reported start site. An antisense construct designed to target this newly identified transcript caused a pronounced increase in the expression of *CDKN2A*, but had a much less profound effect on *CDKN2B*.[31] In conjunction with various other experiments, the authors concluded that the transcript encoded upstream of the previously reported start site of *CDKN2BAS* controls *CDKN2A* silencing by cis recruitment of polycomb group complex proteins, and in addition functions as an architectural matrix to retain these proteins at the target site. Interestingly, a recent study, which performed genome wide expression profiling

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in healthy individuals who were homozygous for the common and the rare allele of rs1333049, showed that the nature of the genes and pathways linked to *CDKN2BAS* transcript levels comprised genes which control cellular proliferation.[30] These studies did not functionally analyse exons 1—19, but a second antisense construct designed to target exon 3 silenced *CDKN2A* to a much lesser extent than the antisense construct upstream of exon 1.[31] Although the function of this transcript was not studied in detail, it indicates that different *CDKN2BAS* transcripts may have different functions. Currently, 10 alternative spliced variants were reported in different tissues as diverse as lung, brain, heart, vascular epithelial tissues, testis, and blood.[30 45 46] They may have specific functions in their tissue specific context, additional or alternative to the observed negative control of *CDKN2A*. Two studies measured the expression of *CDKN2BAS* spliced transcript *NR_003529*, spanning exons 17 to 18, and found slightly decreased expression which was associated with the risk allele in healthy individuals.[30 32]

A study on individuals with clinically suspected CHD revealed that *NR_003529* expression was significantly increased in individuals with atherosclerotic plaque burden, and correlated *ANRIL* transcript levels with severity of atherosclerosis.[47] Our primers for the quantification of *CDKN2BAS* transcript levels were designed to detect *NR_003529* transcripts, and we found *NR_003529* upregulation by bacterial infection. The correlation of *CDKN2BAS* expression with bacterial infection and increased *CDKN2BAS* expression in individuals with atherosclerotic plaque burden add to the theory of inflammatory components in the pathophysiology of CHD. Considering the recent finding on the ability of the 5′ transcript of *CDKN2BAS* to recruit and retain regulatory proteins, the functions of the proximal and considerably long sequences of *CDKN2BAS* might well have a similar mechanistic function, but may also be able to act in trans.

Theories concerning the actual disease mechanism that is mediated by the causative risk variants of *CDKN2BAS* remain speculative, as the exact nature of the causative variants and the target proteins or genes await elucidation. They possibly differ between CHD and periodontitis. Here, we present findings which link the pathogenicity of *P gingivalis* and its contribution to rapid periodontal degradation to a biological mechanism that is mediated by *CDKN2BAS*, that may directly act in cis on the negative regulation of *CDKN2A* and the downstream lying genes of the *CDKN2A* regulation cascade; or additionally, may act on other, as yet unknown, trans-acting regulatory proteins with unidentified genetic targets. Taking into consideration the correlation of increased *ANRIL* transcript levels with the severity of atherosclerosis,[52] this link might also join bacterial infection to atherosclerotic plaque burden by a potentially regulatory mechanism that is mediated by *ANRIL* transcription.

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