Original Article

Genetic Evidence for *PLASMINOGEN* as a Shared Genetic Risk Factor of Coronary Artery Disease and Periodontitis

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Background—Genetic studies demonstrated the presence of risk alleles in the genes *ANRIL* and *CAMTA1/VAMP3* that are shared between coronary artery disease (CAD) and periodontitis. We aimed to identify further shared genetic risk factors to better understand conjoint disease mechanisms.

Methods and Results—In-depth genotyping of 46 published CAD risk loci of genome-wide significance in the worldwide largest case–control sample of the severe early-onset phenotype aggressive periodontitis (AgP) with the Illumina Immunochip (600 German AgP cases, 1448 controls) and the Affymetrix 500K array set (283 German AgP cases and 972 controls) highlighted *ANRIL* as the major risk gene and revealed further associations with AgP for the gene *PLASMINOGEN* (*PLG*; rs4252120: $P=5.9\times10^{-5}$; odds ratio, 1.27; 95% confidence interval, 1.3–1.4 [adjusted for smoking and sex]; 818 cases; 5309 controls). Subsequent combined analyses of several genome-wide data sets of CAD and AgP suggested *TGFBRAP1* to be associated with AgP (rs2679895: $P=0.0016$; odds ratio, 1.27 [95% confidence interval, 1.1–1.5]; 703 cases; 2.143 controls) and CAD ($P=0.0003$; odds ratio, 0.84 [95% confidence interval, 0.8–0.9]; n=4117 cases; 5824 controls). The study further provides evidence that in addition to *PLG*, the currently known shared susceptibility loci of CAD and periodontitis, *ANRIL* and *CAMTA1/VAMP3*, are subjected to transforming growth factor-β regulation.

Conclusions—*PLG* is the third replicated shared genetic risk factor of atherosclerosis and periodontitis. All known shared risk genes of CAD and periodontitis are members of transforming growth factor-β signaling.
(*Circ Cardiovasc Genet*. 2015;8:159-167. DOI: 10.1161/CIRCGENETICS.114.000554.)

Key Words: coronary artery disease ■ genetic association studies ■ periodontitis ■ plasminogen

Periodontitis is a chronic inflammatory disease of the oral cavity. The inflammation is elicited by the oral microbial biofilm that leads to gingival bleeding, pocket formation, alveolar bone loss, and eventually tooth loss as final outcome.[1] Periodontitis affects human populations worldwide at prevalence rates of 11% for the severe forms.[2] It is largely classified into the subforms chronic periodontitis (CP) and aggressive periodontitis (AgP). Whereas chronic periodontitis is mostly observed in adults and is characterized by a slow progress of the disease, AgP is the most early-onset and most extreme phenotype and found in young individuals aged <35 years. AgP is diagnosed based on rapid destruction of the alveolar bone and is comparatively rare in the general population with prevalence rates ≈0.1%. Shared with coronary artery disease (CAD), smoking, obesity, and mellitus diabetes contribute strongly to periodontitis[3–5] and both diseases are characterized by chronic inflammation.[6] Likewise, epidemiological studies demonstrated an association between the presence of CAD and periodontitis,[7] which is dependent on the severity of periodontal disease.[8] We previously showed that CAD and periodontitis are genetically related by ≥2 genetic susceptibility loci, the long noncoding antisense RNA (lncRNA) *ANRIL* (*CDKN2BAS*) at chromosome 9p21.3[9,10] and a

Received February 11, 2014; accepted October 16, 2014.

The Data Supplement is available at http://circgenetics.ahajournals.org/lookup/suppl/doi:10.1161/CIRCGENETICS.114.000554/-/DC1.

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conserved noncoding element within *CAMTA1* upstream of *VAMP3*.[11] Molecular biological data placed ANRIL and VAMP3 into a regulatory network that integrates glucose and fatty acid metabolism with immune response,[11,12] providing evidence for a mechanistic link between CAD, periodontitis, obesity, and inflammation. The impairment of these pathways by genetic factors may be a common pathogenic denominator of CAD and periodontitis.

Clinical Perspective on p 167

In the present study, we aimed to identify further putatively shared genetic risk factors of CAD and periodontitis to better understand the common genetic architecture of these diseases. In the first stage, we genotyped 46 CAD risk loci, which gave published evidence for genome-wide association with CAD.[13] These chromosomal regions were genotyped on the Illumina Immunochip[14] and the Affymetrix 500K Genotyping Array Set[15] using cases of AgP. For AgP it is thought that smoking, obesity, diabetes mellitus, and age have a minor role in disease development and patient samples are considered enriched with genetic risk factors that are located in genes involved in the pathogenesis of periodontitis.

In a second stage of this study, we combined several genome-wide data sets of CAD and AgP from Germany and the UK and propose a significant association within *TGFBRAP1* for replication in future enlarged case–control samples of AgP.

Materials and Methods

Study Population

Details on the study populations are given in Table 1, in the Data Supplement, and in the Tables I to III in the Data Supplement. Written informed consent was obtained from all participants. The recruitment and experimental protocols were approved by the institutional ethics review board and data protection authorities.

Genotyping

Immunochips were genotyped and analyzed as described in Ref. 16 and Affymetrix 500K arrays as described in Refs. 15, 17, and 18. We included all single-nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) ≥5% and at a Hardy–Weinberg equilibrium for controls >5%, and with a call rate of >95%. SNP rs1981458 and rs17514846 (*FURIN*), rs4252120 (*PLASMINOGEN*) and rs2679895 (*TGFBRAP1*) were also genotyped on 384-well plates using TaqMan assays hCV11947689, hCV1244341, hCV11225947, and hCV27295110, respectively (Applied Biosystems) as described.[10]

Analysis of Candidate Genes

Twenty-seven chromosomal regions, which gave published evidence of genome-wide association with CAD, were genotyped using the Immunochip (Illumina) in 600 German AgP cases and 1443 population representative German controls (Table 1).[13] In addition, 18 further CAD risk loci of genome-wide significance were genotyped using the Affymetrix 500K Arrays set with 283 German AgP cases and 979 German controls.[15] See Tables IV and V in the Data Supplement for gene names, SNPs, genotypes, and association statistics of the associated genetic regions. Potential associations that were observed for genotype data of the immunochip were validated in silico in an independent case–control panel of 159 Dutch AgP cases and 679 population representative Dutch controls as described in [16]. Potential associations that were observed for genotype data of the 500K array genome-wide association study (GWAS) data were replicated using a panel of 424 German AgP cases and 3628 German controls and validated using 159 Dutch AgP cases and 352 periodontal healthy Dutch controls, as described in Table 1.

Statistical Analysis

SNP imputation was performed in silico with the BEAGLE v.3.1.141 software package[19] and HapMap3 reference haplotypes from the Central Europeans of Utah (CEU) cohorts[20] to predict missing autosomal genotypes in silico. We subsequently analyzed only those SNPs that could be imputed with moderate confidence (r^2>0.3) and had a minor allele frequency >1% in cases or in controls as previously described.[21] Genotypes were analyzed using the software PLINK v2.[22] Significance of association was assessed using χ^2 tests. Power calculations were performed using PS Power and Sample Size Calculations software.[23] Linkage disequilibrium measures were calculated with Haploview 4.1.[24] Logistic regression analysis was performed to adjust for possible confounding of the covariates smoking and sex in the R statistical environment.[25] For the regression analyses, smoking was coded as a binary variable according to whether a patient or control person had ever smoked. The genetic models considered were (1) an allelic model (genotypes aa, aA, and AA coded as 0, 1, and 2, respectively), (2) a dominant model (genotypes coded as 0, 1, and 1), and (3) a recessive model (genotypes coded as 0, 0, and 1). Thus, each logistic model included 3 independent variables: 2 binary covariates (sex and smoking), plus the genetic variable that was either binary (dominant or recessive genetic model) or considered continuous (allelic model). The dependent variable was always the case/control status, coded as 1 or 0, respectively.

Details on the cutoff criteria for the selection of SNPs to be taken into replication and the statistical analyses and imputation methods are described in the Data Supplement.

Quantitative Real-Time Polymerase Chain Reaction

Transcript levels were analyzed by quantitative real-time reverse transcription polymerase chain reaction. Primers and conditions are described in the Data Supplement.

Cell Culture Conditions and Transforming Growth Factor-β Stimulation

Human gingival fibroblastic primary cells were derived from 3 different individuals during dental surgery. The cells of each donor were cultured in DMEM containing 1% penicillin/streptomycin and 10% heat inactivated fetal bovine serum. For each of the 3 different donors, 6 cell cultures were grown independently as biological replicates. After reaching 50% to 60% confluence, the medium of 3 cultures of each donor was replaced by fresh medium supplemented with 20 ng/mL transforming growth factor (TGF)-β for 48 hours. Three cultures of each donor continued to grow for 48 hours in replaced fresh medium that lacked the TGF-β supplement. For each biological replicate, the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicates. For each donor, the mean for 3 replicates was calculated and standard 1- and 2-tailed *t* tests were performed. For *ANRIL*, *C11ORF10*, and VAMP3, where the effect directions were unknown, 2-tailed tests were performed. For *ADIPOR1* and *CDKN2B*, where the effect directions after TGF-β stimulation were known from literature, a 1-tailed test was performed.

Results

Candidate Gene Association Study of Known CAD Risk Loci in AgP

Immunochip

In an explorative step of the first stage of this study, 27 established CAD risk loci of genome-wide significance were genotyped with the Immunochip. Two loci, *ANRIL* and *FURIN,* suggested association with periodontitis and fulfilled the

Table 1. Assignment of the Analysis Populations to the Different Stages of the Study

	Candidate Gene Study						Joint GWAS Analysis			
	Stage 1			Stage 2			Stage 1	Stage 2	Stage 3	Stage 4
	Immunochip		TaqMan (*FURIN*; rs1981458, rs17514846)	Affymetrix 500K	TaqMan (*PLG;* rs4252120)		Affymetrix 500K			TaqMan (*TGFBRAP1;* rs2679895)
Samples	Exploration (D)	Validation (NL)	D	Exploration (D)	Replication (D)	Validation (NL)	Exploration AgP/CAD (D)	Replication CAD (D)	Validation CAD (UK)	Replication AgP (D)
Cases										
AgP (German)	600	…	600/628	283	…	…	283	…	…	…
AgP (Dutch)	…	164	159	…	424	159	…	…	…	427
MI (GerMIF-I)	…	…	…	…	…	…	…	970	…	…
MI (GerMIF-II)	…	…	…	…	…	…	1222	…	…	…
MI (WTCCC)	…	…	…	…	…	…	…	…	1925	…
Sum cases	600	164	600/159	283	424	159	283/1222	970	1925	427
Controls										
Popgen (SPCs)	471	…	471	500	…	…	500	…	…	…
Popgen (PopCONs)	977	…	…		…	…	…	…	…	…
Popgen (BSPs)	…	…	449	479	…	…	479*	…	…	…
KORA	…	…	3028†/3259‡	…	3259	…	820	1644	…	…
MICK	…	…	…	…	…	…	…	…	…	1292
Periofree (Munich)	…	…	…	…	423	…	…	…	…	222
UMCG (NL)	…	679	…	…	…	…	…	…	…	…
ACTA (NL)	…	…	352	…	…	352	…	…	…	…
WTCCC (UK)	…	…	…	…	…	…	…	…	2936	…
Sum controls	1443	679	3948/4179/352	979	3682	352	979/1299	1644	2936	1514

Detailed characteristics of the individual samples are given in the Data Supplement. ACTA indicates Academic Centre for Dentistry Amsterdam; AgP, aggressive periodontitis; BSP, blood donors special phenotyped; CAD, coronary artery disease; GerMIF, German Myocard Infarct Family Study; KORA, kooperative Gesundheitsforschung in der Region Augsburg; MI, myocardial infarction; MICK, Metabolic Intervention Cohort Kiel; PopCON, biobank popgen controls; SPC, special phenotpyed controls; UMCG, University Medical Center Groningen; and WTCCC, Wellcome Trust Case-Control Consortium.

*Part of the GWAS of AgP and the GERMIF-II; †metabochip; and ‡OmniExpress array. For details on the study samples please refer to the supplemental materials.

preassigned significant threshold of $P<6\times10^{-3}$ (Table VI in the Data Supplement; Table 2). The associations of *ANRIL* with AgP were in accordance with our previous studies.[9,10] To show the independence of this association from established covariates, we adjusted rs1981458 for smoking and sex by logistic regression analysis. Because data on smoking were not available for 977 of the popgen PopCon control sample, we excluded these controls from the analysis. To keep sufficient statistical power (SP) for analysis of this low frequency variant, we added another 449 German blood donors (BSP; sample of Blood donors Special Phenotyped) from the biobank popgen and additional 3028 population representative German controls from the biobank KORA (Kooperative Gesundheitsforschung in der Region Augsburg) , for which data on smoking and sex were available. Before and after adjustment, this association remained significant (Table 2).

SNP rs1981458 is located 55 bp downstream of the common GWAS lead SNP rs17514846 (MAF=47%; HapMap CEU) and is not associated with CAD in the CARDIoGRAMplusC4D Consortium, encompassing >63 000 CAD cases and >130 000 controls.[13] rs17514846 was not included on the Immunochip. To test whether rs17514846 was also associated with AgP, we genotyped the 600 German AgP cases, which were also used for the immunochip and 4179 German healthy controls from the biobanks popgen and KORA (Table 1; Table I in the Data Supplement). The association of rs17514846 was not significant here.

Affymetrix 500K

Genetic risk loci of CAD[13] that were represented on the Immunochip by ≤2 SNPs were analyzed using Affymetrix 500K arrays (Table V in the Data Supplement). Of those 28 loci, only associations within the gene *PLG* fulfilled the preassigned selection criteria. The best association signals were shown by *PLG* SNP rs4252135 (MAF=28.3%, HapMap CEU) and the *PLG* GWAS lead SNP of CAD, rs4252120 (Table VII in the Data Supplement). After covariate adjustment for sex and smoking, the association of rs4252120 with AgP was significant with $P_{allelic}$=0.00019 (odds ratio [OR], 1.46; 95% confidence interval [CI], 1.20–1.78; Table 2). In a further sample of 424 less severe German AgP cases and 3682 controls, this association slightly missed significance (Table 1). After adjustment for the covariates smoking and sex, the association was nominally significant under the dominant genetic model but not under the allelic model (Table 2). To increase the SP, both German samples were pooled (n=706 cases, 4957 controls). After adjustment for sex and smoking, the association

Table 2. AgP Association Statistics for the Best Associated SNPs at *FURIN* and *PLG*

					Cases					Controls				
Gene, SNP	Population	SNP	*P* Values	OR (95% CI)	11% (n)	12% (n)	22% (n)	Sum	MAF (%)	11% (n)	12% (n)	22% (n)	Sum	MAF (%)
FURIN, rs1981458	German	Unadjusted	0.00581	1.34 (1.1–1.7)	76.2 (457)	22.5 (135)	1.3 (8)	600	12.6	81.6 (1168)	17.5 (251)	0.9 (13)	1432	9.7
		Adjusted (smoking, sex)	0.0115*	1.28 (1.1–1.6)	76.2 (457)	22.5 (135)	1.3 (8)	600	12.6	81.0 (3197)	17.7 (700)	1.3 (51)	3948†	10.2
	Dutch	Unadjusted	0.96088	1.01 (0.7–1.5)	81.1 (133)	17.7 (29)	1.2 (2)	164	10.1	81.2 (550)	17.6 (119)	1.2 (8)	677	10.0
PLG, rs4252120	GWAS (German)	Unadjusted	0.00018‡	1.44 (1.2–1.8)	40.4 (114)	47.5 (134)	12.1 (34)	282	35.8	51.7 (659)	40.9 (521)	7.5 (95)	1275	27.9
		Adjusted (smoking, sex)	0.00019	1.46 (1.2–1.8)	…	…	…	…	…	…	…	…	…	…
	Replication (German)	Unadjusted	0.05524	1.2 (1.0–1.4)	44.3 (188)	45.1 (191)	10.6 (45)	424	33.1	49.1 (1809)	41.9 (1541)	9.0 (332)	3682	29.9
		Adjusted (smoking, sex)	0.0571‖	1.18 (1.0–1.4)	…	…	…	…	…	…	…	…	…	…
	Pooled (German)	Unadjusted	0.00024	1.25 (1.1–1.4)	42.8 (302)	46.0 (325)	11.2 (79)	706	34.2	49.8 (2468)	41.6 (2062)	8.6 (427)	4957	29.4
		Adjusted (smoking, sex)	0.00071	1.25 (1.1–1.4)	…	…	…	…	…	…	…	…	…	…
	Replication (Dutch)	Unadjusted	0.01158	1.44 (1.1–1.9)	42.8 (68)	44.0 (70)	13.2 (21)	159	35.2	53.7 (189)	37.8 (133)	8.5 (30)	352	27.4
		Adjusted (smoking, sex)	0.00964	1.51 (1.1–2.1)	…	…	…	…	…	…	…	…	…	…
	Pooled all (German, Dutch)	Adjusted (smoking, sex)	5.9×10^{-5}	1.27 (1.1–1.4)	42.8 (370)	45.7 (395)	11.6 (100)	865	34.4	50.1 (2657)	41.4 (2195)	8.6 (457)	5309	29.3

CI indicates confidence interval; MAF, minor allele frequency; OR, odds ratio; and SNP, single-nucleotide polymorphism.

*The *P* value for the dominant model is *P*=0.00625; OR, 1.35 (95% CI, 1.1–1.7).

†The control sample that was used for the covariate adjustment consisted of 922 North-German controls (popgen) and an additional sample of 3026 South-German controls (KORA).

‡A total of 296 additional popgen blood donor controls were used in the TaqMan genotyping (n=1275) compared with Affymetrix 500K genotyping (n=979) to increase the statistical power. This explains the discrepancy with the *P* value in the text of the article (*P*=0.00059; see also Table VII in the Data Supplement).

‖*P* value is given for the dominant genetic model. The *P* value for the dominant model is *P*=0.0336; OR, 1.27 (95% CI, 1.0–1.6).

of rs4252120 was significant with P=0.00071 and a genetic effect of OR of 1.25 and 95% CI of 1.1 to 1.4.

We validated the association in the smaller Dutch AgP panel of high severity and found the same genetic effect direction (Table 2). Next, to increase the SP, the German and Dutch samples were pooled (865 cases, 5309 controls) and adjusted for sex and smoking. The association became more significant with $P_{allelic}=5.9\times10^{-5}$ (Table 2).

Combined GWAS Analysis of CAD and AgP

In the second stage of this study, SNPs that suggested association with CAD in imputed genotypes of GWAS data of the German Myocard Infarct Family Study (GerMIFS)-II were tested if they showed a similar level of significance in imputed genotypes of the GWAS on AgP. A total of 30 383 SNPs showed a P value of <0.05 in the GerMIF-II GWAS and 99 154 SNPs showed a P value of <0.05 in the AgP GWAS. Of these SNPs, 1182 SNPs were shared between both GWAS (Figure 1). Next, these SNPs were tested for association in a second imputed GWAS of CAD, the GerMIFS-I. At the same significance threshold, 128 SNPs showed nominal association. To further reduce the number of suggestive SNP associations, we strengthened the selection criteria. SNPs were selected, which showed a genetic effect in the same direction between the 2 CAD GWAS samples at a level of significance of P<0.01, and additionally SNPs, which showed a level of significance at a P<0.01 between the replication panel GerMIFS-I and the AgP GWAS sample, regardless of the effect direction (Figure 1). Twenty-one SNPs fulfilled these selection criteria (Table VIII in the Data Supplement). These associations were subsequently replicated in the Wellcome Trust Case-Control Consortium CAD GWAS sample (Table VIII in the Data Supplement). Five SNPs were located within *ANRIL* and 2 within *TGFBRAP1* (Table 3). Whereas the shared association of *ANRIL* with AgP and CAD had repeatedly been observed by us and others,[9,10,16,26] an association of *TGFBRAP1* with CAD or periodontitis has not been reported previously. The ORs of the 2 *TGFBRAP1*-associated SNPs rs920217 and rs2679895 were identical in the 3 CAD samples (OR<0.9), which was opposite to the genetic effect in the AgP sample (Table 3). The MAFs of both SNPs were similar in all CAD and AgP control samples. After adjustment of rs2679895 for the covariates smoking and sex, the effect of the rare allele of rs2679895 remained significantly associated with AgP ($P_{allelic}$=0.0169; OR, 1.33; 95% CI, 1.05–1.68; Table 4).

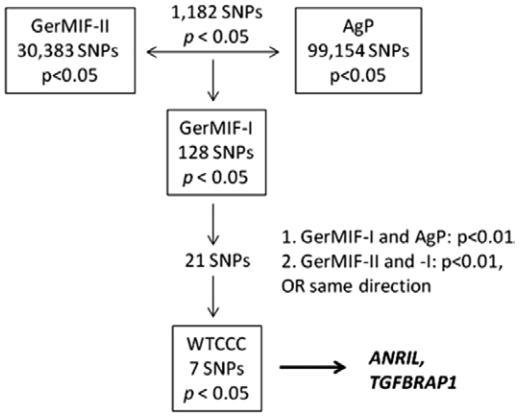

Figure 1. Study design of the combined GWAS analyses. To generate hypotheses on candidate genes that are shared in the pathogenesis of coronary artery disease and periodontitis, the GWAS were combined as illustrated. The individual GWAS samples are published in Refs. 15, 17, and 18. AgP indicates aggressive periodontitis; GerMIF, German Myocard Infarct Family Study; SNP, single-nucleotide polymorphism; and WTCCC, Wellcome Trust Case-Control Consortium.

To localize the association with *TGFBRAP1* more precisely, all SNPs that were represented on the Affymetrix 500K arrays were tested for association with the CAD-Wellcome Trust Case-Control Consortium sample and the AgP GWAS sample (28 SNPs; Figure 2). Notably, a GWAS on progression of AIDS (Acquired Immune Deficiency Syndrome) reported an association with rs1020064, which was not genotyped in our samples. This SNP is located ≈1000 bp upstream of rs2576742 (Figure 2),[27] which was genotyped in our samples. SNP rs2576742 showed a similar level of association as rs2679895 (CAD-Wellcome Trust Case-Control Consortium: P=0.0056; OR, 0.87 [95% CI, 0.79–0.96] and AgP: P=0.0041; OR, 1.36 [95% CI, 1.10–1.68]). Analysis of the linkage disequilibrium structure of rs2679895, rs2576742, and the AIDS-associated SNP rs1020064 in a sample of German population representative controls (n=211), for which genotypes of these 3 SNPs were available, showed complete linkage (r^2>0.98;

Table 3. Summary Statistics of Candidate SNP Associations of the Joint GWAS Analysis of Coronary Artery Disease and AgP (Affymetrix 500K)

		GerMIF-II* (German)		GerMIF-I* (German)		WTCCC† (British)		AgP† (German)	
Gene	SNP	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)
ANRIL	rs10965212	0.0028	1.19 (1.06–1.33)	4.39E–04	1.22 (1.09–1.37)	6.94E–10	1.29 (1.2–1.4)	8.26E–04	1.38 (1.14–1.66)
	rs10965215	0.0061	1.17 (1.05–1.31)	3.40E–04	1.23 (1.10–1.37)	4.32E–10	1.30 (1.2–1.4)	0.0011	1.37 (1.13–1.65)
	rs564398	1.22E–04	0.80 (0.71–0.90)	7.12E–04	0.82 (0.73–0.92)	1.65E–08	0.79 (0.7–0.9)	5.26E–04	0.71 (0.59–0.86)
	rs10965219	0.0016	1.20 (1.07–1.34)	1.11E–03	1.21 (1.08–1.35)	3.94E–11	1.32 (1.2–1.4)	2.82E–04	1.42 (1.17–1.71)
	rs6475606	5.25E–07	1.33 (1.19–1.48)	1.11E–05	1.29 (1.15–1.44)	2.44E–14	1.37 (1.3–1.5)	2.56E–04	1.42 (1.18–1.71)
TGFBRAP1	rs920217	0.0435	0.87 (0.76–0.996)	0.0095	0.84 (0.73–0.96)	0.0262	0.89 (0.81–0.99)	0.02427	1.30 (1.03–1.63)
	rs2679895	0.0435	0.87 (0.76–0.996)	0.0095	0.84 (0.73–0.96)	0.0083	0.87 (0.79–0.97)	0.0081	1.36 (1.08–1.70)

AgP indicates aggressive periodontitis; CI, confidence interval; GerMIF, German Myocard Infarct Family Study; OR, odds ratio; SNP, single-nucleotide polymorphism; and WTCCC, Wellcome Trust Case-Control Consortium.

*Imputed data; †genotyped data.

Table 4. Summary Statistics of *TGFBRAP1* SNP rs2679895 for the Different Aggressive Periodontitis Samples After Covariate Adjustments

	German 1*		German 2†		German Pooled†		Dutch*		Turkish*	
Genetic Model	*P* Value (AIC)	OR (95% CI)	*P* Value (AIC)	OR 5% CI)	*P* Value (AIC)	OR (95% CI)	*P* Value (AIC)	OR (95% CI)	*P* Value (AIC)	OR (95% CI)
Allelic	0.0169 (1074)	1.33 (1.05–1.68)	0.0443 (1562)	1.22 (1.004–485)	0.0016 (2692)	1.27 (1.09–1.47)	0.0305 (588)	0.68 (0.48–0.96)	0.0332 (113)	0.44 (0.20–0.92)
Recessive	n.s.	1.49 (0.82–2.66)	0.0123 (1560)	1.80 (1.13–2.84)	0.0077 (2695)	1.62 (1.13–2.31)	0.0269 (584)	0.63 (0.41–0.94)	0.0126 (111)	0.29 (0.11–0.76)

AIC indicates Akaike's Information Criterion; CI, confidence interval; and OR, odds ratio.
*Adjusted for smoking and sex.
†Adjusted for smoking.

Figure I in the Data Supplement). This is supported by the data of the HapMap CEU reference population.

Associations of *TGFBRAP1* SNP rs2679895 With AgP in Samples of Different Geographical and Ethnical Background

We replicated the association of rs2679895 with AgP in 427 German AgP cases of a less severe phenotype (Table 1; Table III in the Data Supplement). Because the KORA control sample was part of the GerMIF-I and II studies and the popgen controls were part of the 500K Array GWAS on AgP, we used an independent sample of population representative German controls from the Metabolic Intervention Cohort Kiel (n=1292) and additional 222 periodontitis-free controls. In the replication, rs2679895 showed similar association with AgP as was observed in the first German AgP sample (Table 5 and see Table 6 for allele frequencies and genotypes). Because the Metabolic Intervention Cohort Kiel control sample was selected for diabetes mellitus–free men, adjustment for potential sex effects would have introduced a bias. Therefore, we adjusted for smoking only. The association remained significant (Table 4). To increase the SP, we pooled all German AgP cases and controls and adjusted for smoking. After adjustment of this largest sample, the allelic model showed an association with *P*=0.0016; OR, 1.27 (95% CI; 1.09–1.47; Table 4).

We tested the association of rs2679895 with the smaller Dutch AgP sample (Table III in the Data Supplement). In this sample, SNP rs2679895 was significant (Table 5), but with a reversed OR compared with the discovery sample, also after adjustment for smoking and sex (Table 4).

Finally, we tested the association of SNP rs2679895 in a Turkish AgP sample (91 cases, 75 healthy controls; Table 4). Here, the association was significant, also with a reversed OR compared with the German case–control samples (Table 4).

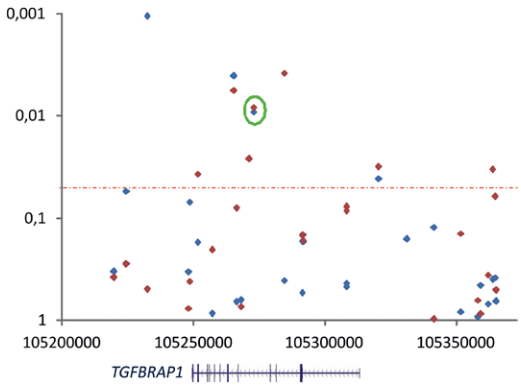

Figure 2. $-\log_{10} P$ values of the genotyped single-nucleotide polymorphisms (SNPs) of *TGFBRAP1* aligned to the chromosomal map. The top diagram shows the nominal $-\log^{10} P$ of the 26 SNPs, which were genotyped at this chromosomal region in the German aggressive periodontitis (AgP) GWAS sample and the Wellcome Trust Case-Control Consortium (WTCCC) coronary artery disease (CAD) GWAS sample (Affymetrix 500K). The *P* values are aligned to the genomic SNP position (National Center for Biotechnology Information build 36). The panel below shows the chromosomal position of *TGFBRAP1.* SNPs that were genotyped in the AgP sample are shown in blue, SNPs from the WTCCC CAD sample are shown in red. SNP rs2679895 is depicted by the green circle. Dashed horizontal red line, Nominal significance threshold of *P*=0.05. Three SNPs passed this threshold in both samples: rs2576742 (in complete linkage disequilibrium with the AIDS (Acquired Immune Deficiency Syndrome)-associated SNP rs1020064, see Figure I in the Data Supplement), rs2679895, and rs2033727 (depicted below in bold letters). SNP-IDs from **left** to **right**: rs4851743, rs17636399, rs17687727, rs4851752, rs2460255, rs17030722, rs3816133, rs2576742, rs17688608, rs3792048, rs2679895, rs17689220, rs1985599, rs873738, rs893249, rs17695158, rs17638586, rs2033727, rs2576776, rs880427, rs6543286, rs10193943, rs6543288, rs10190186, rs6543289, and rs6753046.

Shared Risk Loci of CAD and Periodontitis Are Regulated by TGF-β

TGFBRAP1 associates with the TGFBR complex[28] and was reported to act as an inhibitor of TGF-β signaling.[29] *PLG* was reported to activate TGF-β signalling[30,31] and *FURIN* was also shown to be regulated by TGF-β.[32] Recently, we showed that *ANRIL* regulates the distant genes *ADIPOR1*, *VAMP3,* and *C11ORF10* and an earlier study demonstrated that the expression of *ADIPOR1* was negatively regulated by TGF-β.[33] This indicated that genes that are related to the disease pathogenesis of both CAD and AgP may act together in the same biological pathway. We tested if, in addition to *PLG*, *TGFBRAP1, FURIN*, and *ADIPOR1* and also the expression of *ANRIL, VAMP3,* and *C11ORF10* were regulated by TGF-β signaling. After TGF-β stimulation of human gingival fibroblasts, the transcriptional isoform of *ANRIL* that was previously shown to be positively correlated with *ADIPOR1*,

Table 5. Summary Statistics of *TGFBRAP1* Single-Nucleotide Polymorphism rs2679895 in 3 Aggressive Periodontitis Case–Control Samples

Germany/Austria		Dutch/Irish		Turkish	
P Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)
0.0099	1.26 (1.06–1.49)	0.0260	0.71 (0.52–0.96)	0.0450	0.59 (0.35–0.99)

CI indicates confidence interval; and OR, odds ratio.

Table 6. Allele Frequencies and Genotypes for *TGFBRAP1* Single-Nucleotide Polymorphism rs2679895 in the AgP and CAD Case–Control Samples

		Cases					Controls				
Disease	Population	11% (n)	12% (n)	22% (n)	MAF%	Sum	11% (n)	12% (n)	22% (n)	MAF%	Sum
AgP	Germany	50.0 (138)	42.4 (117)	7.6 (21)	28.8	276	59.5 (374)	35.1 (221)	5.4 (34)	23.0	629
	Germany, Austria (replication)	53.9 (230)	37.7 (161)	8.4 (36)	27.3	427	59.5 (901)	34.9 (529)	5.5 (84)	23.0	1.514
	NL, Ireland (validation1)	66.5 (135)	30.0 (61)	3.4 (7)	18.5	203	57.7 (205)	36.1 (128)	6.2 (22)	24.2	355
	Turkey (validation2)	68.1 (62)	27.5 (25)	4.4 (4)	18.1	91	53.3 (40)	38.7 (29)	8.0 (6)	27.3	75
CAD	GerMIF-I† (Affy 500K)	62.3 (604)	32.9 (319)	4.9 (47)	21.3	970	56.7 (909)	37.8 (606)	5.6 (89)	23.4	1604
	GerMIF-II† (Affy 500K)	62.8 (767)	32.4 (396)	4.8 (59)	21.0	1.222	59.6 (765)	34.0 (437)	6.4 (82)	24.4	1284
	WTCCC (Affy 500K)	64.4 (1.240)	31.5 (607)	4.1 (78)	19.8	1.925	60.5 (1.776)	34.9 (1.025)	4.6 (135)	22.1	2.936

AgP indicates aggressive periodontitis; CAD, coronary artery disease; GerMIF, German Myocard Infarct Family Study; MAF, minor allele frequency; and WTCCC, Wellcome Trust Case-Control Consortium.

VAMP3, and *C11OF10* expression[11] was significantly reduced (P=0.0209). *VAMP3* expression was also significantly reduced (P=0.0023), whereas the expression of *C11ORF10* did not significantly change before and after 48 hours of TGF-β stimulation (Figure 3).

Discussion

In the past, various risk alleles of *ANRIL* gave repeated evidence for association with CAD as well as with periodontitis.[9,10,18,34–37] In both parts of the current study, the candidate gene and joint GWAS analyses, we identified several variants within the coding region of *ANRIL* as risk factors shared between both diseases, showing the power of both approaches.

We identified the CAD GWAS lead SNP rs4252120, located within the coding region of *PLG*, to be associated with AgP, replicated this association, and demonstrated independence of the genetic effect from the confounding factors smoking and sex. In the context of this study, it is of interest that the PLG-plasmin system has an important function for degradation of tissue barriers and cell migration.[38] Various pathogenic bacteria were found to bind human PLG on bacterial PLG receptors,[39,40] which turns them into proteolytic organisms,[41] for example, the common periodontal pathogen *Porphyromonas gingivalis* is able to activate human PLG expression and to inactivate human plasmin inhibitors, causing uncontrolled plasmin activity.[41] Along with other periodontal pathogens, *P. gingivalis* was also detected in thrombi at the sites of acute myocardial infarction.[42] We could not replicate the observed association of SNPs at *FURIN*. However, the associated rare allele of rs1981458 is a less frequent variant with MAF=10% and the Dutch replication panel lacked SP to reject or accept the null-hypothesis of no association. Yet, the function of FURIN makes it an interesting candidate for future research into putatively shared disease mechanisms, because FURIN, similar to PLG, is required for many pathogens to become fully pathogenic, as previously reviewed.[43]

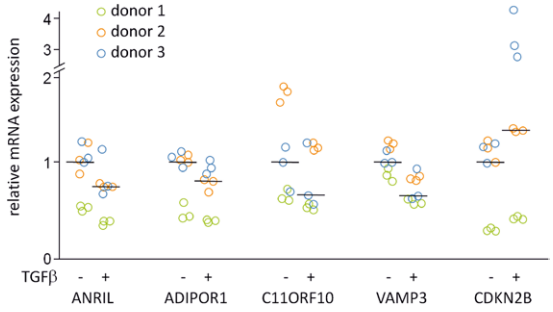

Figure 3. Relative expression of *ANRIL*, *ADIPOR1*, *C11ORF10*, *VAMP3*, and *CDKN2B* in response to transforming growth factor (TGF)-β stimulation. Human gingival fibroblasts from 3 different donors were stimulated with TGF-β for 48 hours (20 ng/mL). The figure shows the relative changes of transcript levels for each replicate of each donor (median, horizontal lines). After TGF-β stimulation, transcript levels were significantly reduced for *ANRIL* (P=0.02089), *ADIPOR1* (P=0.03649), and *VAMP3* (P<0.002344). The transcript levels of C11ORF10 and *CDKN2B* did not significantly change after TGF-β stimulation (C11ORF10: P=0.1623; CDKN2B: P=0.1068).

In the second stage of this study, we found an association of SNP rs2679895 within *TGFBRAP1* in 3 independent CAD case–control samples and 4 independent AgP samples and demonstrated independence of the genetic effect from the confounding factors smoking and sex. In all CAD samples, the genetic effect of the association was in the same direction but in opposite direction to the 2 large German AgP samples. Similarly, the genetic effect of rs2679895 was in opposite direction in the Dutch–Irish AgP and Turkish AgP compared with the German AgP samples. Between the different control groups, the MAFs were similar, indicating that the observed effect was transmitted by the cases. We did not identify a putative causative disease associated variant at this genetic locus. It is possible that the opposite genetic effects observed in the German compared with the Dutch and Turkish AgP samples could be because of different linkage disequilibrium structures between these samples, which have different geographical and historical as well as ethnical background, respectively. Variants with both the same and opposite effects in the same pairs of different diseases have been reported before.[44] Replication studies in larger samples and finemapping of this genetic locus are required to fully elucidate the nature of the observed associations at *TGFBRAP1*. Our data suggest TGF-β signaling to have a role in the shared pathogenesis of CAD and periodontitis. This is in accordance with various studies that indicate that TGF-β signaling may represent a common site of intersection between the various downstream pathologies of obesity-induced complications[37] that comprise CAD and periodontitis. Interestingly, the identification of the adipokine

plasminogen activator inhibitor-1, an inhibitor of the PLG-plasmin system, to be strongly upregulated in visceral adipose depots in obesity also suggests a link of the PLG-plasmin system and obesity.

In conclusion, we give evidence that *PLG* is a shared genetic susceptibility factor of CAD and AgP. We further propose *FURIN* and *TGFBRAP1* as good candidates for future replication studies in larger samples. The current study emphasizes the role of TGF-β signaling in the shared pathogenesis of CAD and periodontitis.

Acknowledgments

The contributions of the following researchers and technicians are gratefully acknowledged: Michael Wittig, Ines Spitzer, Ilona Urbach, Tanja Wesse, and Tanja Kacksteen and of the information specialist of the biobank popgen Lukas Tittmann. We also thank Robert Häsler for his good advices and helpful support.

Sources of Funding

This work was supported by a research grant of the "Research Center Inflammation Medicine" of the Medical Faculty, Christian-Albrechts-University (Dr Schaefer), by a grant of the Deutsche Forschungsgemeinschaft (KFO208; Drs Schaefer, Bochenek, Dommisch, and Jepsen), by a grant of the Dutch Society of Periodontology (Drs Loos and Schaefer) and by a research priority grant from the University of Amsterdam (Dr Loos). The popgen 2.0 network is supported by a grant from the German Ministry for Education and Research (01EY1103). The KORA research platform (KORA, Cooperative Research in the Region of Augsburg) was initiated and financed by the Helmholtz Zentrum M€unchen – German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research and by the State of Bavaria.

Disclosures

None.

Appendix

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CLINICAL PERSPECTIVE

Periodontitis is an inflammatory disease of the oral cavity caused by bacteria forming a biofilm on the gingiva. For the severe forms, periodontitis affects human populations worldwide at prevalence rates of 11%. Strong evidence of associations between the presence of periodontitis and coronary artery disease (CAD) was derived from multiple randomized clinical trials. Shared risk alleles of periodontitis and CAD within the 2 genes *ANRIL* and *CAMTA1/VAMP3* had been described. To better understand the conjoint disease mechanisms, this study aimed to systematically identify further shared genetic risk variants. In-depth genotyping of all major CAD risk loci of genome-wide significance and combined analyses of several genome-wide data sets of CAD and of the severe phenotype aggressive periodontitis highlighted *ANRIL* as the major shared risk gene of both diseases and established the gene *PLASMINOGEN* (*PLG*) as a newly discovered shared susceptibility gene (rs4252120, $P=5.9\times10^{-5}$; odds ratio, 1.27; 95% confidence interval, 1.3–1.4). The PLG-plasmin system has an important function for degradation of tissue barriers and in previous studies various oral pathogenic bacteria were found to bind human PLG, which turns them into proteolytic organisms. These pathogens had been detected in thrombi at the sites of acute myocardial infarction and are discussed to directly or indirectly induce inflammatory responses with possible impacts on the pathogenesis of CAD. The study further provides evidence that all currently shared genetic risk loci of CAD and periodontitis are subjected to transforming growth factor-β regulation. These findings underpin future hypothesis-driven research into the inflammatory disease mechanisms of CAD.

SUPPLEMENTAL MATERIAL

Description of the study populations

Genetic population structure of Germany and recruitment area of the German cases

Population genetic differentiation within the German population indicated a minor degree of population structure.[1] This conclusion was supported by a previous study, which implied that even genetic differences between geographically adjacent European subpopulations are small.[2] Another study analysed the distribution of 'best' genetic-matching partners between the European subpopulations and the inter-individual variability in terms of the uniqueness of the 'best' genetic-matching partner and observed that the within-subpopulation genetic variation is much greater than the between European subpopulation variation.[3] These studies make the use of population representative controls from North- and South-Germany appropriate to match cases, which were sampled across Germany, as recently described.[4-8] The AgP cases were recruited across Germany, to their largest parts at the following university dental clinics from North-Germany (Kiel), West-Germany (Bonn, Frankfurt am Main, Münster), the geographical center of Germany (Hannover), East-Germany (Dresden) and South-Germany (Munic and Würzburg). The Austrian cases were recruited at the university dental clinic of Vienna.

Phenotype definitions of Aggressive periodontitis

Inclusion criteria for the AgP cases, which were genotyped with the Immunochip were ≥2 teeth with 30% alveolar bone loss under the age of 35 and no diabetes. Only individuals of German and Austrian ethnicity were included, judged upon the location of both parental birthplaces and German family names. They were first described in.[9]

AgP cases, which were genotyped with the Affymetrix 500K array sets were a subset of cases that were genotyped with the immunochip. However, the GWAS subsample was selected for a particular strong severity with ≥2 teeth with 50% alveolar bone loss under the age of 35 years

and no diabetes. Only individuals of German ethnicity were included. They were first described in.[10]

The German AgP cases, which were used for the replication of the Affymetrix 500 K arrays consisted in parts (N=317) of the cases, which were genotyped on the Immunochip and of additional German cases (N=107) that had not previously been genotyped. The inclusion criteria and geographical origin of these additional cases were ≥2 teeth with 30% alveolar bone loss under the age of 35 and no diabetes.

The Dutch AgP cases sample was also selected for a particular strong severity, which was comparable to the German cases that were genotyped on the Affymetrix 500K arrays, with ≥2 teeth with 50% alveolar bone loss under the age of 35 years and no diabetes. These cases were recruited by the ACTA (Academisch Centrum Tandheelkunde) Amsterdam within the geographical region of Holland and were first described in.[10]

A set of full-mouth dental radiographs or orthopantomograms were available from the German, Austrian and Dutch cases for confirmative periodontal bone scoring. Aspects of the medical history, health status and smoking habits (current-, former-, non-smoker or smoker) were recorded by a questionnaire.

The Irish cases were classified with AgP if at least one third of all teeth exhibited ≥50% bone loss (full-mouth dental radiographs available). In cases where no radiographs were available, ≥1/3 of all teeth had to have clinical attachment loss of ≥5mm. The Irish cases were described in.[11]

The Turkish AgP cases were selected according to the criteria accepted by the American Academy of Periodontology.[12] They were recruited at Hacettepe University, Ankara and Kocaeli University. Evaluation of all cases consisted of personal, family, medical and dental history, full-mouth periapical radiographs, and periodontal examination. Exclusion criteria included history of hepatitis or HIV infection, medical/systemic disorders/diseases (such as

diabetes), immunodefiency, pregnancy or lactation, long-term usage of anti-inflammatory drugs, requirement of antibiotic prophylaxis, and younger than 18 years of age at the time of participation in the study. Each individual's age, sex, smoking habits and sociodemographic variables were recorded. This sample was first described in[9]

AgP controls

Controls, which were genotyped with the Immunochip were recruited by the North-German biobank popgen and were randomly identified on the basis of the population registry of Schleswig-Holstein. A subset of 471 controls (SPCs; **S**pecial **P**henotyped **C**ontrols) underwent an additional physical examination at the popgen facilities to obtain information on the general health status. Informations on the oral health status and physical risk factors (e.g. smoking, diabetes) were obtained from a questionnaire that was completed during medical consultation. Additionally, a clinical checkup was performed. They were first described in[13]

Controls, which were genotyped with the Affymetrix 500K arrays consisted of 500 SPC controls from popgen and of 479 additional blood donors (BSPs; **B**lood donors **S**pecial **P**henotyped), which were recruited by the biobank popgen from the Blood Service of the University Hospital Schleswig-Holstein, Kiel. They underwent the same additional examinations as the SPCs and were first described in.[13]

German controls, which were used for the replication of the SNP associations at *FURIN* and *PLG*, participated in the South-German population based MONICA/KORA Augsburg survey that was conducted as part of the German National Genome Research Network (NGFN). This survey represents a gender- and age stratified random sample of all German residents of the Augsburg area (Bavaria, South-Germany) and consists of individuals 25 to 74 years of age, with about 300 subjects for each 10-year increment. The population was studied by physical examination, blood testing, and a standardized interview including medical history, physical

activity, medication, and personal habits and was first described in.[14]

For the replication of the associations of *TGFBRAP1* SNP rs2679895 controls from the Metabolic Intervention Cohort Kiel (MICK) were used (N=1,292). These controls were selected by age from the general population via the population registry of the region surrounding the city of Kiel, Germany. Inclusion criteria were males, aged 42-68 years and absence of self-reported diabetes. Exclusion criteria were intake of hormones or lipid-lowering medication, surgery of the intestinal tract in the last 3 months or other alterations of the gastrointestinal tract, active cancer, chronic renal or liver disease, anemia and alcohol abuse. They were first described in.[5, 15] For the replication of the associations of *TGFBRAP1*SNP rs2679895 additional 222 periodontitis-free controls were used, which had been recruited from the Blood Service of the Bavarian Red Cross (Munich, Southern-Germany) at the time of the analysis. For the genotyping of *PLG* SNP rs4252120, this blood donor sample was also used in addition to the KORA control sample. At the time of the genotyping of *PLG* SNP rs4252120, this sample had been enlarged to 423 controls. The absence of periodontal disease was proven for these blood donor control subjects by a trained dentist using to the following criteria: (1) a minimum of 22 teeth *in situ*, (2) ≤1 site with probing pocket depth ≥3mm, (3) lack of any kind of furcation involvement at any tooth. This control sample has been described in.[16]

Dutch controls, which were genotyped with the Immunochip were recruited by the Blood service of the University Medical Center Groningen (UMCG), Holland, The Netherlands. Dutch controls, which were used for the replication of the SNPs at *PLG* and *TGFBRAP1* were recruited by ACTA via the Bloodbank Sanquin, Amsterdam, Holland, The Netherlands. They were first described in.[10] In a self report they indicated to be free of any periodontal diseases. No further clinical check-up was performed. All Dutch controls were generally healthy.

The Turkish controls were generally healthy and had no history and sign of periodontal disease, i.e. no obvious clinical inflammation and no bleeding on probing and were collected at

Hacettepe University, Ankara and Kocaeli University, Turkey and were first described in.[9] The population representative controls and blood donors were generally regarded to be free of AgP, as the prevalence of AgP is very low with an estimated occurrence of <0.1%.

CHD cases

German Myocard Infarct Family study (GerMIFS) I: 875 case subjects in the GerMIFS-I were South-German individuals who had myocardial infarction before the age of 60 years and at least one first degree relative with premature coronary artery disease. The German Myocard Infarct Family study (GerMIFS) II comprised 1,222 South-German patients with a validated myocardial infarction and a documented early age of onset <60 years. A positive family history for CAD was documented in 726 (59.4%) of patients. Patients of the GerMIFS-I and –II were identified following their admission for acute treatment of MI or in cardiac rehabilitation clinics. They were described in.[5]

The WTCCC CAD cases were nationally recruited unrelated individuals of European white ethnicity from UK with premature CAD (a validated history of either MI or coronary revascularization before age 66 years) and one or more first degree relatives with CAD. They were described in.[17]

CHD controls

The controls of the GerMIFS I were 1,644 German controls subjects who participated in the population based South-German MONICA/KORA Augsburg study in the years 1994/95 and in a follow-up of this survey in the years 2004/05 that was conducted as part of the German National Genome Research Network (NGFN).[14] This survey represents a sex- and age stratified random sample of all German residents of the Augsburg area and consists of individuals 25 to 74 years of age, with about 300 subjects for each 10-year increment. The

population was studied by physical examination, blood testing, and a standardized interview including medical history, physical activity, medication, and personal habits. By questionnaire, 22% of the individuals reported a positive parental family history for MI. The controls of GerMIFS II were population-based controls derived from the MONICA/KORA Augsburg survey S4 1 (n=820) and the popgen blood donor sample (BSP, n=478).

The WTCCC CAD controls were 2,936 unrelated individuals from the '1958 British Birth Cohort' and UK Blood Services. They were described in.[17]

Statistical Analysis

Parallel analyses of multiple SNPs require a decrease of the significance threshold to avoid false-positive findings. Over-conservative correction for multiple testing increases the possibility of false-negative findings. To avoid false rejection of associations of SNPs with intermediate frequency or effects, we started by identifying SNPs meeting a less-stringent cut-off for association ($P<6\times10^{-3}$). To eliminate random SNP associations, to be taken into replication, an associated genetic region had to be additionally flanked by a significant marker at $P<0.01$ or by two flanking markers at $P\leq0.05$. If a single GWAS lead SNP was genotyped, a significance threshold of $P<6\times10^{-3}$ was sufficient to be taken into replication.

Candidate Genes

27 chromosomal regions, which gave published evidence of genome-wide association with CAD were genotyped in 600 German AgP cases and 1,441 German population representative controls using the Immunochip (Illumina) (*ABO, ADAMTS7, ANKS1A, ANRIL, APOE-ApoC1, chr11 ZNF259_APOA5, COL4A1-COL4A2, CXCL12, CYP17A1-CNNM2-NT5C2, FURIN, gene desert KCNE2, LDLR, LPL, PHACTR1, SH2B3, SLC22A3-LPAL2-LPA, SLC22A4-SLC22A5, TCF21, TRIB1*; **Supplemental Table 4**).[4] 15 loci were covered by ≥ 5

SNPs, twelve loci were only represented by the CAD-GWAS lead SNP or by an additional second SNP. To improve genotype coverage, these twelve loci (*ABO, APOE-ApoC1, COL4A1-COL4A2, CXCL12, gene desert KCNE2, GUCY1A3, MIA3, MRAS, PHACTR1, SLC22A3-LPAL2-LPA, TCF21, WDR12*) were additionally analyzed using genotype data of 283 German AgP cases and 1,330 German controls using the Affymetrix 500K Arrays set[10]. In addition, 18 further CAD risk loci of genome-wide significance were analyzed using genotype data of this array set (*7q22, EDNRA, FLT1, HDAC9, HHIPL1, KCKNK5, KIAA1462, LIPA, PCSK9, PDGFD, PLG, PPAP2B, RAI1-PEMT-RASD1, SMG6, UBE2Z, VAMP5-VAMP8-GGCX, ZC3HC1, ZEB2-ACO74093.1*; **Supplemental Table 5**).[4]

Quantitative Real-Time PCR

Transcript levels were analyzed by quantitative real-time reverse transcription-PCR (qRT-PCR) using the following conditions and primers: 50°C 2 min; 95°C 10min; 95°C 15sec; 60°C 1min; 45 cycles. Differences in transcript levels between stimulated and unstimulated samples were calculated using two-tailed T-Tests.

ADIPOR1 fwd: TCGGACTTTTTCCAAACTGG, rev: GCTGTGGGGAGCAGTAGAAG; ***C11ORF10*** fwd: AGATATACCAGCCCAGTG, rev: AGTGTACTTGGTAGAGGTGA; ***CDKN2B*** fwd: CACCATGAAGCGAAACACAG, rev: TCCATCGGAAGATTCGTAGC; ***ANRIL*** fwd: GGA CAT TGG ACA AAA ACA CAG A, rev: GCA GGT ATC ATT CTC CTC AA AT; (Ex13)

VAMP3 fwd: GTGGCCAGCTGTGAGGAGAA, rev: GGGTTACATGGGTCTGGGCA;

GAPDH (housekeeping gene) fwd: TGACATCAAGAAGGTGGTGA, rev: TGTCGCTGTTGAAGTCAGAG

Cell culture

Human gingival fibroblastic primary cells (hGFCs) were derived from different patients during dental surgery and were cultured in DMEM containing 1% Penicilline/Streptomycin, and 10% heat inactivated FCS. After reaching 50-60% confluence, the medium was replaced by fresh medium supplemented with TGF-β (20ng/ml) and hGFCs were cultured in the new medium for 48 hours before harvesting. Each biological replicate represented cells from three different patients. For each patient's cells the experiment was replicated three times. Accordingly, the values represent the average of five independent experiments each performed in technical triplicates. For quantification of the relative expression, qPCR was performed using the SYBR Green PCR Master Mix (Life Technologies). Amplification was performed with the 7900 HT Fast Real-Time PCR System (Applied Biosystems) allowing direct detection of the PCR product by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA.

Supplemental Table 1. Characteristics of the AgP study population used for the candidate gene study - stage 1 (Immunochip)

	Immunochip				Co-variate adjustment *FURIN* (rs1981458)				Co-variate adjustment *FURIN* (rs17514846)			
	German		Dutch		German				German			
Samples	SPC, popCON	Cases	UMCG (blood donors)	Cases	SPC	BSP	KORA*	Cases$^{\Delta}$	SPC	BSP	KORA#	Cases$^{\Delta}$
Male	701	225	404	43	255	264	1,465	225	255	264	1,579	225
Female	747	324	152	113	216	185	1,563	324	216	185	1,680	324
Unknown	0	51	123	3	0	0	0	51	0	0	0	51
Smoked	228	345	-	121	228	270	1,693	345	228	270	1,668	345
Never smoked	243	201	-	38	243	174	1,335	201	243	174	1,378	201
Unknown	977	54	679	0	0	5	0	54	0	5	213	54
total	1,448	600	679	159	471	449	3,028	600	471	449	3,259	600

Supplemental Table 2. Characteristics of the AgP study population used for the association tests of *PLG* SNP rs4252120

	Exploration		Replication *PLG*			Validation *PLG*	
	German		German			Dutch	
	SPC, BSP	Cases	KORA	Periofree (MÜ)	cases	ACTA	cases
Male	706	98	1,579	281	177	151	42
Female	569	185	1,680	142	208	198	117
unknown	0	0	0	0	39	3	0
Smoked	700	128	1,668	50	217	141	126
Never smoked	575	155	1,378	373	133	201	31
unknown	0	0	213	0	74	10	2
total	1,275	283	3,259	423	424	352	159

Supplemental Table 3. AgP case-control samples used for replication and validation of *TGFBRAP1* SNP rs2679895

	Replication			Validation 1		Validation 2	
	Germany			Netherlands/Ireland*		Turkey	
	Controls (MICKs)	Controls (PD-free, MÜ)	Cases	ACTA (NL)	Cases	Controls (PD-free)	Cases
Male	1,292	129	180	154	61	10	25
Female	0	69	210	198	142	23	31
unknown	0	24	37	3	0	42	37
Smoked	166	10	220	140	158	7	20
Never smoked	744	87	135	205	43	24	35
unknown	352	125	72	10	2	44	38
total	1,292	222	427	355	203	75	93

*44 AgP cases from Ireland were added to increase the statistical power.

Supplemental Table 4. Genes and SNPs, which were genotyped on the Immunochip in the AgP case-control sample

Chr.	Gene	GWAS CAD lead SNPs	analyzed SNPs	Association	Aver. SNP distance kb (aver. deviation)
1	*IL6R*	rs4845625		no	3.21 (2.36)
1	*SORT1*	rs602633, rs599839	6	no	1.46 (1.28)
1	*MIA3*	rs17464857	1	no	
2	*ABCG5-ABCG8*	rs6544713	7	no	1.56 (1.43)
2	*APOB*	rs515135	1	no	
2	*WDR12*	rs6725887	1	no	
3	*MRAS*	rs9818870	1	no	
4	*GUCY1A3*	rs7692387, rs13139571	2	no	
5	*SLC22A4-SLC22A5*	rs273909	332	no	0.87 (0.74)
6	*ANKS1A*	rs12205331, rs17609940	744	no	1.1 (1.35)
6	*TCF21*	rs12190287	1	no	
6	*PHACTR1*	rs9369640, rs12526453	1	no	
6	*SLC22A3-LPAL2-LPA*	rs3798220, rs2048327	1	no	
8	*TRIB1*	rs2954029	50	no	1.2 (1.49)
8	*LPL*	rs264	7	no	2.91 (2.01)
9	*ABO*	rs579459	12	no	1.88 (2.63)
9	***ANRIL***	rs1333049, rs3217992	11	**yes**	8.82 (4.62)
10	*CYP17A1-CNNM2-NT5C2*	rs12413409	84	no	3.84 (3.32)
10	*CXCL12*	rs501120, rs2047009	1	no	
11	*ZNF259_APOA5*	rs9326246	15	no	1.14 (1.62)
12	*SH2B3*	rs3184504	134	no	2.43 (2.06)

13	*COL4A1-COL4A2*	rs4773144, rs9515203	1	no	
15	*ADAMTS7*	rs7173743	282	no	0.79 (0.73)
15	***FURIN***	rs17514846	5	**yes**	3.47 (1.66)
19	*LDLR*	rs1122608	11	no	5.81 (2.71)
19	*APOE-ApoC1*	rs2075650, rs445925	2	no	
21	gene desert *KCNE2*	rs9982601	1	no	

Aver.=average

Supplemental Table 5. Genes, which were genotyped on the Affymetrix 500K arrays in the AgP case-control sample due to incomplete coverage of the Immunochip

Chr.	Gene	CAD-GWAS lead SNP	SNPs at this region on Immunohip
1	*MIA3*	rs17464857	1
1	*PCSK9*	rs11206510	0
1	*PPAP2B*	rs17114036	0
2	*APOB*	rs515135	1
2	*ZEB2-ACO74093.1*	rs2252641	0
2	*VAMP5-VAMP8-GGCX*	rs1561198	0
2	*WDR12*	rs6725887	1
3	*MRAS*	rs9818870	1
4	*EDNRA*	rs1878406	0
6	*KCKNK5*	rs10947789	0
6	***PLG***	rs4252120	0
6	*TCF21*	rs12190287	1
6	*PHACTR1*	rs9369640, rs12526453	1
6	*SLC22A3-LPAL2-LPA*	rs3798220, rs2048327	1
7	*ZC3HC1*	rs11556924	0
7	*7q22*	rs12539895	0
7	*HDAC9*	rs2023938	0
10	*CXCL12*	rs501120, rs2047009	1
10	*KIAA1462*	rs2505083	0
10	*LIPA*	rs2246833, rs11203042	0
11	*PDGFD*	rs974819	0
13	*COL4A1-COL4A2*	rs4773144, rs9515203	1
13	*FLT1*	rs9319428	0
14	*HHIPL1*	rs2895811	0
17	*RAI1-PEMT-RASD1*	rs12936587	0
17	*UBE2Z*	rs15563, rs46522	0
17	*SMG6*	rs2281727, rs216172	0
21	gene desert *KCNE2*	rs9982601	1

Supplemental Table 6. Association statistics and Immunochip genotype data of the AgP case-control sample for the significant loci .

									cases				controls			
Chr.	**Gene**	**SNP**	**Position (bp)**	**Distance (kb)**	**p-value**	**OR**	**L95**	**U95**	**11**	**12**	**22**	**MAF**	**11**	**12**	**22**	**MAF**
9	*ANRIL*	rs1412829	22,033,926	24.726	0.00147	0.80	0.70	0.92	229	272	96	38.86	452	702	287	44.27
		rs4977756	22,058,652	8.891	0.00042	0.78	0.68	0.90	240	268	91	37.56	471	685	285	43.55
		rs1412832	22,067,543	5.861	0.00078	0.78	0.67	0.90	300	247	52	29.30	630	621	190	34.73
		rs1333040	22,073,404	12.651	0.00329	0.81	0.71	0.93	226	265	108	40.15	431	714	292	45.16
		rs10757274	22,086,055	7.758	0.00634	1.21	1.06	1.38	159	274	156	49.75	428	728	285	45.04
		rs1333042	22,093,813	12.146	0.00027	1.29	1.12	1.47	149	270	180	52.59	404	721	299	46.31
		rs2383207	22,105,959	8.518	0.00033	1.28	1.12	1.47	134	282	183	54.09	387	727	327	47.92
		rs10757278	22,114,477	0.87	0.00949	1.20	1.05	1.37	157	290	150	49.41	430	724	285	44.96
		rs1333048	22,115,347	0.156	0.00279	1.23	1.07	1.41	151	277	166	51.26	412	729	300	46.11
		rs1333049	22,115,503	6.573	0.00927	1.20	1.05	1.37	160	284	154	49.50	427	729	284	45.03
		rs2383208	22,122,076	N.A.	0.99720	1.00	0.84	1.19	400	181	18	18.11	952	443	38	18.11
15	*FURIN*	rs4932370	89,205,709	5.304	0.84210	0.99	0.86	1.13	241	286	73	36.00	587	661	193	36.33
		rs3759929	89,211,013	1.647	0.89590	0.99	0.86	1.14	207	314	78	39.23	532	681	228	39.45
		rs4932178	89,212,660	4.949	0.66090	0.97	0.84	1.11	224	292	82	38.13	536	690	215	38.86
		rs1981458	89,217,609	1.986	0.00581	1.34	1.09	1.66	457	135	8	12.58	1168	251	13	9.67
		rs4526996	89,219,595	N.A.	0.00799	1.33	1.08	1.64	458	133	8	12.44	1176	252	13	9.65

bp=basepair, OR=odds ratio, L95=lower confidence interval 95%, U95= upper confidence interval 95%, MAF=minor allele frequency

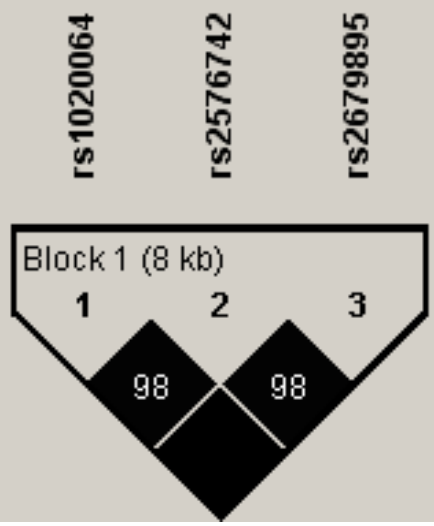
Supplemental Table 7. Association statistics for the SNPs at *PLG*, which were genotyped on the Affymetrix 500 K Arrays in the AgP case-control sample (282 cases, 979 controls)

SNP	BP	P	OR	L95	U95
SNP_A-4215186	161.042.911	NA	NA	NA	NA
SNP_A-4266065	161.043.175	0.6235	0.9531	0.7869	1.155
SNP_A-4227819	161.048.937	0.2535	0.8424	0.6275	1.131
SNP_A-4253070	161.052.820	0,0751	0.5709	0.4317	0.7551
SNP_A-4255364	161.053.036	0.2735	1.139	0.9024	1.437
SNP_A-2206482	161.057.248	0.8347	1.186	0.2381	5.911
SNP_A-2059692	161.057.653	0.00073	1.407	1.154	1.717
SNP_A-4201898	161.057.769	0.7919	0.9644	0.7364	1.263
SNP_A-1883064	161.058.838	0.3072	0.8714	0.669	1.135
SNP_A-2012233	161.063.223	0.9633	1.024	0.376	2.787
SNP_A-2012234	161.063.366	0.00047	1.424	1.167	1.736
SNP_A-4297397	**161.063.598**	**0.00059**	**1.416**	**1.6**	**1.727**
SNP_A-1958850	161.064.326	0.837	1.183	0.2375	5.895
SNP_A-1879517	161.066.793	0.9948	1.005	0.2076	4.867
SNP_A-2186918	161.071.446	0.4469	0	0	NA
SNP_A-1786089	161.072.284	0.00067	1.414	1.158	1.726
SNP_A-2112886	161.073.070	0.00062	1.422	1.162	1.741
SNP_A-2306423	161.073.517	0.0039	1.347	1.1	1.649
SNP_A-4288436	161.074.222	0.00034	1.445	1.181	1.768
SNP_A-2115357	161.074.514	NA	NA	NA	NA
SNP_A-1827463	161.076.221	NA	NA	NA	NA

BP=basepair, OR=odds ratio, L95=lower confidence interval 95%, U95= upper confidence interval 95%, NA= not applicable, bold=CAD GWAS lead SNP rs4252120

Supplemental Table 8. Association Statistics of SNPs, which were replicated with the WTCCC CAD sample

				AgP		GerMIF-II		GerMIF-I		WTCCC	
locus	SNP	Chr.	Position (bp)	p	OR	p	OR	p	OR	p	OR
SRBD1	rs2344657	2	45,584,043	0.0217	0.61	0.0086	0.72	0.0082	0.69	0.0164	0.75
TGFBRAP1	rs920217	2	105,271,094	0.0006	0.74	0.0371	0.87	0.0093	0.84	0.0262	0.89
	rs2679895	2	105,272,912	0.0008	1.35	0.0383	0.87	0.0093	0.84	0.0083	0.87
Intergenic (*OTOS*)	rs4853991	2	240,765,904	0.0081	0.78	0.0483	1.15	0.0016	1.25	n.s.	n.s.
ALAS1	rs352169	3	52,211,802	0.0086	0.81	0.0442	1.12	6.89E-04	1.22	n.s.	n.s.
LINC01091	rs3113390	4	124,985,565	0.0066	1.251	0.0042	1.18	0.0011	1.211	0.4932	0.97
Intergenic (*PRELID2*)	rs17391049	5	144,440,645	0.0002	0.73	0.0327	1.15	0.0020	1.23	0.7041	1.02
Intergenic (*PER4*)	rs10225720	7	9,575,097	0.0050	1.29	0.0010	1.24	0.0077	1.20	n.s.	n.s.
AGPAT6	rs11996670	8	41,574,736	0.0364	1.19	0.0065	0.86	5.68E-04	0.82	n.s.	n.s.
	rs13248554	8	41,593,447	0.0143	0.82	0.0046	1.18	4.36E-05	1.27	n.s.	n.s.
	rs7010327	8	41,599,666	0.0315	0.84	0.0044	0.85	3.54E-04	0.81	n.s.	n.s.
ANRIL	rs10965212	9	22,013,795	0.0240	1.20	0.0028	1.19	4.39E-04	1.22	6.94E-10	1.29
	rs10965215	9	22,019,445	0.0249	1.20	0.0061	1.17	3.41E-04	1.23	4.32E-10	1.30
	rs564398	9	22,019,547	0.0408	0.84	0.0001	0.80	7.12E-04	0.82	1.65E-08	0.79
	rs10965219	9	22,043,687	0.0150	0.82	0.0015	1.20	0.0011	1.21	3.94E-11	1.32
	rs6475606	9	22,071,850	0.0471	0.85	0.0000	1.33	1.11E-05	1.29	2.44E-14	1.37
	rs7860403	9	70,861,066	0.0002	0.55	0.0376	1.32	0.0054	1.48	n.s.	n.s.
Intergenic (*POLR1D*)	rs9512814	13	27,180,256	0.0082	1.26	0.0133	0.86	0.0043	0.84	n.s.	n.s.
Intergenic (*NEDD4L*)	rs8095739	18	53,698,643	0.0260	0.84	0.0050	1.17	1.64E-04	1.24	n.s.	n.s.
	rs4941164	18	53,701,458	0.0210	1.20	0.0069	1.17	1.64E-04	1.24	0.6582	1.02
	rs4121751	18	53,710,464	0.0070	1.25	0.0217	1.14	2.63E-05	1.28	n.s.	n.s.



Supplemental Figure 1. LD structure of the AIDS associated SNP rs1020064 and the CAD and AgP associated SNPs rs2576742 and rs2679895

Numbers in the black diamonds are r^2-values, calculated with 211 German population representative controls

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Circulation
Cardiovascular Genetics

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Circ Cardiovasc Genet. 2015;8:159-167; originally published online December 2, 2014;
doi: 10.1161/CIRCGENETICS.114.000554
Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1942-325X. Online ISSN: 1942-3268