Integration mouse and human genetic studies for mapping periodontitis susceptibility

Nashef A[1], Qabaja R[1], Salaymeh Y[2], Botzman M[3], Munz M[4,5],Dommisch H[4], Krone B[6], Hoffmann P[7],[8] Wellmann J[9], Laudes M[10], Berger K[9], Kocher T[11], Franke A[12], Offenbacher S[13], Lieb W[14], Divaris K[13,15], Mott R[16], Gat-Viks I[3], Wiess E[17], Schaefer A[4*], Iraqi FA[2*] and Haddad YH[1*].

[1]Department of Prosthodontics, Hadassah Faculty of Dental Medicine, Hebrew University Jerusalem, Israel.

[2] Department of Clinical Microbiology and Immunology, Sackler Faculty of Medicine, Tel-Aviv University, Israel.

[3] Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel.

[4] Department of Periodontology and Synoptic Medicine, Institute for Dental and Craniofacial Sciences, Charité – University Medicine Berlin, Germany.

[5] Institute for Integrative and Experimental Genomics, University Medical Center Schleswig-Holstein - Campus Lübeck, Germany

[6]Institute of Medical Informatics, Biometry and Epidemiology, University Clinic Essen, Germany

[7]Institute of Human Genetics, University of Bonn, Germany

[8]Germany und Human Genomics Research Group, Department of Biomedicine, University Hospital of Basel, Switzerland

[9] Institute of Epidemiology and Social Medicine, University of Münster, Germany.

[10]Clinic of Internal Medicine, University Clinic Schleswig-Holstein, Kiel, Germany

[11]Unit of Periodontology, Department of Restorative Dentistry, Periodontology, Endodontology, Preventive Dentistry and Pedodontics, Dental School, University Medicine Greifswald, Germany

[12]Institute of Clinical Molecular Biology, Christian-Albrechts-University, Kiel, Germany

[13]University of North Carolina-Chapel Hill, School of Dentistry, Department of Periodontology, Chapel Hill, United States of America

[14] Institute of Epidemiology, Biobank popgen, Christian-Albrechts-University, Kiel, Germany

[15]University of North Carolina-Chapel Hill, Gillings School of Global Public Health, Department of Epidemiology, Chapel Hill, United States of America

[16]Genetics Institute, University Collage of London, UK

[17]Maurice and Gabriella Goldschleger School of Dental Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

***Equal Contribution**

Corresponding Authors:

Yael Houri-Haddad, Faculty of Dental Medicine, the Hebrew University- Hadassah, P.O. Box 12272, Jerusalem 91120, Israel, Tel: 972-26776195, Fax: 972-2-6429683, Email: **yaelho@ekmd.huji.ac.il**

Fuad A. Iraqi, Department of Clinical Microbiology and Immunology, Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel. Email: fuadi@post.tau.ac.il

Arne Schaefer, Department of Periodontology and Synoptic Dentistry, Institute for Dental and Craniofacial Sciences, Charité – Medical University, Berlin, Germany. Email: arne.schaefer@charite.de

Abstract:

Periodontitis is one of the most common inflammatory human diseases with a strong genetic component. Due to the limited sample size of available periodontitis cohorts and the underlying trait heterogeneity, genome-wide association studies (GWAS) of chronic periodontitis (CP) have largely been unsuccessful in identifying common susceptibility factors. A combination of quantitative trait loci (QTL) mapping in mice with association studies in humans has the potential to discover novel risk loci. To this end, we assessed alveolar bone loss in response to experimental periodontal infection in 25 lines (286 mice) from the Collaborative Cross (CC) mouse population using micro-computerized tomography (µCT) analysis. The orthologous human chromosomal regions of the significant QTL were analyzed for association using imputed genotype data (OmniExpress BeadChip arrays) derived from case-control samples of aggressive periodontitis (AgP; 896 cases, 7,104 controls) and chronic periodontitis (CP; 2,746 cases, 1,864 controls) of Northwest-European and European-American descent, respectively. In the mouse genome, QTL-mapping revealed two significant loci (minus log P-value=5.3; FDR=0.06) on chromosomes 1 (Perl3) and 14

(Perl4). The mapping resolution ranged from ~1.5 Mb to 3 Mb. Perl3 overlaps with a previously-reported QTL associated with residual bone volume in F2 cross and includes the murine gene Ccdc121. Its human orthologue was previously reported to be associated with CP in humans. Use of variation data from the genomes of the CC founder strains further refined the QTLs and suggested seven candidate genes (CAPN8, DUSP23, PCDH17, SNORA17, PCDH9, LECT1, and LECT2). We found no evidence of association of these candidates with the human orthologues. In conclusion, the CC populations enabled mapping of confined QTL that confer susceptibility to alveolar bone loss in mice and larger human phenotype-genotype samples and additional expression data from gingival tissues are likely required to identify true positive signals.

Background:

Periodontitis is a common multifactorial oral disease caused by a dysbiotic oral microbiota and a deregulated host inflammatory response(Hajishengallis, 2014). Apart from external factors, there is clear evidence for the contribution of genetic variation to the susceptibility of periodontitis(Corey et al. 1993). Most suggested susceptibility genes of periodontitis were not met the significance threshold of association or have not been successfully replicated(Vaithilingam et al. 2014), but some genes are considered as true genetic susceptibility factors by giving evidence through independent identification, e.g. *NPY*(Divaris et al. 2013; Freitag-Wolf et al., 2014), *CAMTA1/VAMP3* (Divaris et al. 2012; Bochenek et al. 2013), or repeated replication in independent case-control populations, e.g. *ANRIL*(Ernst et al. 2010) and *GLT6D1*(Schaefer et al. 2010; Hashim et al. 2015).

Chromosomal regions responsible for the genetic variance of complex traits in mice can be mapped as quantitative trait loci (QTL) in experimental populations available for precise study under defined conditions(Iraqi, 2000), and subsequently orthologous genes can be extended successfully to humans. Recently, two QTL studies of periodontitis traits were reported using F_2 population(Shusterman et al. 2013) and recombinant inbred lines (RIL)(Sima et al. 2015) mapping approaches. While the F_2 study reported three QTL (*Perl*1-on chr5, *Perl*2-on chr3 and *Perl*3-on chr1) associated with residual bone volume in response to oral bacterial infection(Shusterman et al. 2013), the RIL mapping approach suggested one QTL (iABLL-on chr2) associated with ligature-induced periodontitis phenotype(Sima et al. 2015).

The CC is a novel RIL population, specifically designed for high-resolution mapping QTL(Churchill et al. 2004; Iraqi et al. 2008). It was created from a full reciprocal mating of five classical inbred strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, and NZO/HlLtJ) and three wild-derived strains (CAST/EiJ, PWK/PhJ, and WSB/EiJ) to capture a much greater level of genetic diversity than existing mouse genetic reference populations (GRPs)(Anon 2012). Recently, we showed that the CC lines respond differently to experimental periodontitis induced by mixed infection with *Porphyromonas gingivalis (P.g)* and *Fusobacterium nucleatum (F.n)*(A Shusterman et al. 2013).

Here, we focused to identify genetic variants associated with periodontal disease in humans, by performing for the first time, an integrated analysis of mouse QTL mapping results using the CC population in conjunction with a genetic analysis of the human orthologous chromosomal regions using comprehensive genotype data of two sizeable AgP and CP case-control samples.

Methods:

Mouse production and housing:

286 CC mice, at age 8-12 week old from 25 different CC lines (8- 12 mice on average per line) were provided by the Small Animal Facility, Faculty of Medicine, Tel-Aviv University, Israel. after approval by the Institutional Animal-Care and Use Committee (approved-number: M-08-044). Mice were divided into two groups; infected (138 mice) and control (148 mice). Full details of the development of CC lines were reported previously(Iraqi et al. 2008).

Bacterial cultivation:

P.g strain 381 & *F.n* strain PK 1594 were grown in peptone yeast extract containing Hemin and Vitamin-K (Wilkins Chalgren broth, Oxoid Ltd, UK), in an anaerobic chamber with 85% N_2, 5% H_2 and 10% CO_2 followed by three washes in phosphate-buffered saline (PBS). Bacterial concentrations were measured spectrophotometrically standardized to OD_{650}nm = 0.1 for *P.g*, corresponding to 10^{10} bacteria/ml, and OD_{660}nm = 0.26 for *F.n*, corresponding to 10^9 bacteria/ml(Genco et al. 1991; Polak et al. 2009).

Oral Mixed Infection Model:

Initially, mice were treated with sulfamethoxazole (0.8 mg/ml) in drinking water for a continuous period of 10 days, followed by an antibiotic-free period of three days, before oral application of mixed culture of *P.g* and *F.n* (400ul of 10^9 bacteria/ml per mouse) at days 0, 2 and 4 (control groups were treated with PBS and 2% carboxymethycellulose instead)(Polak et al. 2009). 42 days post infection, mice were sacrificed after complete anesthesia, using two anesthetic materials (Xylisine and Ketamine) and maxillary jaws were harvested for Micro-CT analysis.

Estimation of percentage of alveolar bone loss (PBL) phenotype:

Compact fan-beam-type computerized tomography system (MicroCT40, Scanco Medical, Bassersdorf, Switzerland) was used for quantitative 3-dimensional analysis as described previously(Wilensky et al. 2005). Because the µCT measurement is a destructive procedure, control bone volume (CBV) and residual bone volume due to infection (RBV) cannot be measured in the same mouse. Consequently, it was not possible to obtain estimates of bone loss due to infection for individual animals. However, since the CC mice are at an advanced inbred stages, genetic differences would not present among the mice in a given line, as discussed prviously(Shusterman et al. 2013). Therefore, the percentage of alveolar bone loss (PBL) for each infected mouse was calculated relatively to a control group from the same line.

Statistical analysis and calculation of heritability:

Data analysis performed using SPSS-software version 23. Analysis of variance (ANOVA) was performed to test the differences of response between and within CC lines. We used the ANOVA output of the phenotypic traits to calculate the heritability (H^2)(Iraqi et al. 2014).

Genotyping of mice:

CC lines were genotyped with the mouse diversity array (MDA), which contains 620,000 SNP markers(Yang et al. 2009) and their genome reconstruction was presented(Durrant et al. 2011). The CC lines were re-genotyped at advanced generations with the new 7500 custom-designed mouse universal genotype array (MUGA), which provided the genome architecture of the CC lines(Anon 2012).

QTL Analysis and founder effect:

QTL analysis was performed using R-software including HAPPY.HBREM R-package(Mott et al. 2000). The probability distribution of descent from the eight founders at each interval was calculated and used to test for association between founder haplotype at each locus and PBL phenotype. Permutations of the CC lines between the phenotypes were used to set genome-wide significance thresholds levels and false-discovery rate (FDR) was calculated.

Merge analysis:

We used Sanger mouse genomes database in merge analysis(Yalcin et al. 2005) to test which variants under QTL peak were compatible with pattern of action at the QTL. This takes advantage of the ancestry of the CC to infer the alleles of each CC line based on its genome mosaic (determined from its SNPs) and sequence variation data in the

founder strains. Where a QTL is caused by a single diallelic variant, we expect to have a high chance of testing a very tightly linked tagging SNP with the identical strain distribution pattern, have a higher -logP-value than the 8-way haplotype test in the interval containing the variant, due to the reduction in the dimension of the test.

Mouse QTL and human GWAS integration:

Orthologous human genes were identified using the Ensembl database (www.ensembl.org). Candidate genes that were nominated by merge-analysis and genes within significant loci were selected for genetic analysis in human case-control samples. Additionally, to minimize the likelihood of false negatives, genes at suggestive QTL were analyzed. In addition, genome-wide loci from GWAS studies of different human periodontal disease forms were identified from the GWAS-Catalog(Welter et al. 2014) .

Human study populations:

AgP (Germany). The AgP patients were recruited throughout Germany, the Netherlands, and Austria. Only patients of German and Dutch ethnical background were included, determined by the location of both parental birthplaces and German and Dutch family names. Inclusion criteria for the AgP cases were at least two affected teeth with >30% alveolar bone loss under the age of 35 years, documented by dental radiographs, and no diabetes. The case sample consisted of 896 AgP patients. The study cases were described, previously(Schaefer et al. 2009; Offenbacher et al. 2016).

The AgP control sample consisted of 7,104 controls from Germany and The Netherlands. They were recruited from the Competence Network "FoCus - Food Chain Plus"(Müller et al. 2015), Dortmund Health Study (DHS)(Berger 2012) and the Heinz Nixdorf Recall Studies 1-3 (HNR1-3)(Schmermund et al. 2002). The Dutch control sample consisted of 2,891 (1,453 males 1,438 females), being individuals from the B-Proof Study(Van Wijngaarden et al. 2011).

CP (USA). The American CP cohort was used as described in(Divaris et al. 2012) and consisted of European American participants of the Dental Atherosclerosis Risk In Communities (ARIC) study with moderate CP (n = 1,961 cases; 939 females, 1,022 males; mean age = 63 years) and severe CP (n = 785 cases; 279 females, 506 males; mean age = 64 years). Individuals who were periodontally-healthy or had mild periodontitis were used as controls (N = 1,864 controls; 1,197 females, 667 males; mean age = 62 years)(Hill et al. 1989).

Genotyping and Statistical Tests

All AgP cases and AgP controls were genotyped with OmniExpress arrays on an iScan System (Illumina, USA). SNPs were imputed using 1000G Phase 3 SNPs of Northern Europeans from the HapMap CEPH reference populations (Utah residents with ancestry from northern and western Europe) and the software Impute v2(Howie et al. 2009). After imputation, the control studies were merged using the genetic analysis software Gtools

(http://www.stats.ox.ac.uk/~marchini/software/gwas/gwas.html). Association tests were performed for the AgP case-control sample with SNPTEST v2.5.2(Marchini et al. 2007) assuming an additive genetic model with sex and a binary variable smoking status (never smoked = 1, ever smoked = 0) as covariates. Results of association analyses of the CP sample are publicly available for single makers (SNPs)(Divaris et al. 2013) and gene-centric associations(Rhodin et al. 2014).

Linkage disequilibrium (LD) calculation:

LD between SNPs was analyzed using the 1000GENOMES: phase_3 sub-population CEU (Utah Residents [centre d'etude du polymorphisme humain; CEPH] with Northern and Western Ancestry) as provided by The Ensembl Project (www.ensembl.org).

Results:

Measurement of susceptibility of different CC lines: Our results showed no significant sex effect on bone volume (two- way ANOVA P value>0.05); consequently, both sexes were pooled and treated as same population. The CC strains showed a significant variation in their response to infection (P<0.01). Six lines (IL-26, IL-182, IL-551, IL-711, IL-785, IL-57), showed a significant decrease (P<0.05) in bone volume and considered to be susceptible lines while others were not (Figure 1A). While some lines showed substantial bone loss, other lines showed negative values of percentage of bone loss, indicative of bone formation processes (Figure 1B). One line (IL-519) showed significant increase of bone volume after infection. Heritability estimates of CBV, RBV and PBL were 0.42, 0.45 and 0.33, respectively.

QTL Analysis and founder effect: We identified two significant QTL **(Table1)** associated with percentage of bone loss on chr1 (180-181.5 Mb) and on chr14 (93.5-96.5 Mb) at a significance threshold of minus log P-value=5.3 (FDR = 0.061 in permutation test; **Figure 2A**), and designated as **Pe**riodontitis **R**esistant **L**ocus 3 *(Perl*3) and *Perl*4, respectively. Of note, *Perl*3, which was mapped in the present study, overlaps with the previous suggested *Perl*3-QTL in the F2 population (Chr1:178Mb-181Mb)(Shusterman et al. 2013). In total, 80 genes were underlying *Perl*3 and *Perl*4 (with flanking region of ~0.5 Mb; listed in **Table S1+S2**). In addition, eight suggestive QTLs (defined as P-value<3×10^{-4}; designated as *Perl* 3-10) were mapped on chr1, 14, 15 (two QTL), 14, 2, 7 and 17, respectively (Table1). In total, 1,309 mouse genes were underlying all QTL (Genes are listed in **Tables S3-S10**). At the two most significant QTL (*Perl*3 and *Perl*4), we estimated the effect of each founder haplotype on alveolar bone loss QTL **(Figure 2B).** Both loci on chr1 and chr14 were shown to be less affected by the genetic background of WSB/EiJ (wild-derived strain) rather than the rest of the parental strains.

Association analysis of sequence variants:

The merge analyses for QTL, *Perl*3 and *Perl*4, are shown in Figure 2C+2D. **Table S11** lists the seven candidate genes *(CAPN*8, *DUSP*23, *PCDH*17, *SNORA*17, *PCDH*9, *LECT*1, *LECT*2*)* with the significant merge adjacent SNPs. Both genes, Protocadherin 17 (*PCDH*17) and Protocadherin 9 (*PCDH*9*)* were shown to have a high influence on *Perl*4. While the *PCDH*17 reached the highest significance in the merge analysis (minus logP-value=6.9), *PCDH*9 was found to be the closest gene, with two SNPs with minus logP-value=5.3 and 5.6. Two genes, leukocyte cell derived chemotaxin 1 and 2 *(LECT*1 and *LECT*2*)* had a minus log P-value of ~5.4 and were nominated as suggestive candidate genes. However, the two genes on chr1, Calpain 8 (*CAPN*8*)* and dual specificity phosphatase 23 (*DUSP*23*)* had lower association P-values than in the haplotype mapping (-logP-value 5.04 and 4.91, respectively).

Integration of mouse QTL analysis and human GWAS:

None of seven candidate genes that selected from merge analysis (Table 2) showed significant single SNP-marker associations with human periodontitis in either AgP or CP. However, three genes showed gene-centric associations of periodontal sub-phenotypes; *LECT*1 *(*"orange complex", p= 3.72×10^{-4}), *DUSP*23 (P.g colonization, P=0.037) *and PCDH*17 (P.g colonization, P=0.049) (Rhodin et al. 2014).

The human orthologous genes (31 human orthologous genes– Table S1+S2) of both significant QTL *Perl*3 *and Perl*4 were analyzed for their association to periodontal disease in the AgP and CP GWAS data. We found that one orthologous gene; coiled-coil domain containing 121 genes (*CCDC121)*, which was underling the significant *QTL-Perl*3 (minus log p-value = 5.3 at FDR=0.06), is located 5 Kb upstream of a variant (rs111571364) that was reported as a suggestive risk variant of CP(Teumer et al. 2013) (P-value =8.0×10^{-6}, OR=3.46). However, we could not replicate this association in our AgP and CP samples. In addition, fourteen genes, out of 31 human orthologous genes , have showed gene-centric associations (P<0.05) with periodontal sub-phenotypes; severe CP, 'orange-complex', 'red-complex', *A.a* and *P.g* colonization(Rhodin et al. 2014) (Table S12).

We analyzed the human corresponding genes for additional 1,229 mouse genes underlying suggestive QTLs (defined as P-value<3×10^{-4}). We found that six genes *(NRG*3, *ZNF*579, *FIZ*1, *ZNF*524, *PARK*2, *and PACRG)* showed significant associations with either AgP or CP or both and we suggested them as candidate genes. While three genes *[ZNF*579 (rs149546760, P-value 5.1×10^{-6}*)*, *FIZ1* (rs140900046: P-value 4.4×10^{-6}), and *ZNF*524] showed an association with CP(Teumer et al. 2013), the other three genes (*NRG*3, *PARK*2 *and PACRG*) showed an association with both AgP and CP (either moderate or severe) in our data. Regional association plots of these genes are shown in Figure 3. In total, seven candidate genes were nominated and are summarized in Table 2, based on the integration of mouse QTL and human GWAS.

Discussion:

Previous studies showed the effects of genetic factors on the pathobiology of periodontitis. Because of study limitations of investigations of complex traits in humans, genetic components underlying susceptibility to periodontitis remain largely unknown. In the present study, we performed a combined analysis of high resolution mapping QTL in CC mice and analysis of the orthologous human chromosomal regions using imputed genotype sets of human periodontitis. Our analyses suggest a set of genes to be associated with periodontitis through the combined QTL-GWAS analysis.

Recently, QTL analysis associated with residual bone volume after periodontal infection was reported using F2-population approach(Shusterman et al. 2013) and have successfully mapped two significant QTL with genomic interval of 33Mb on Chr5 and Chr3, and designated *Perl*1 and *Perl*2,respectively, and one suggestive QTL on chromosome 1 with genomic interval of ~3 Mb (*Perl*3). In addition, a suggested genomic region on Chr2 (*iBALL*-QTL Chr2:20-76.8Mb) was, recently reported as associated with ligature-mediated periodontal inflammation(Sima et al. 2015). By using the genetically diverse high CC model, we mapped two significant QTL with ~ 1.5-3 Mb resolution (80 mouse genes/31 orthologous human genes), validating previous reports upon the power of the CC mouse model for high resolution QTL mapping(Durrant et al. 2011). While the previous reported QTL *Perl*1, *Perl*2 and *iABLL* were not replicated in our study, interestingly, our significant *Perl*3 (Chr1) was found to overlap with the previous reported QTL, *Perl*3, which mapped in the F2 approach, showing a stronger, and more precise association. We believe that the discrepancies between the previously reported F2-QTL (*Perl*1 and *Perl*2) and our study are caused by the different genetic diversity of the CC founders compared to the F2 founders and the phenotype definition; while the monitored phenotype in the current study was defined as the percentage of bone loss after periodontal infection compared to a control group using the µ-CT technique, the F2 study was associated with residual bone volume after periodontal infection quantified by µ-CT. We also speculate that iBALL-QTL was not replicated in our study due to the different phenotype definition, disease induction, phenotype quantification techniques and genetic characteristics of the CC-lines compared to the classical RIL.

Merge analysis revealed six SNPs at *Perl*4 interval showed higher P values than the haplotype mapping data, thus these polymorphisms could be considered as candidate causal variants, responsible for the QTL rise. On the other hand, two SNPs on *Perl*3 showed lower values than the haplotype mapping; one possibility other than a false positive observation is that *Perl*3 is raised by a combination of linked variants effect. While three genes, out of seven, showed gene-centric association with periodontal sub-phenotypes, none of human orthologous regions showed significant

single SNP marker association with either CP or AgP. This could be due to differences in the frequency of these variants between CC mice compared to human and/or different regulatory elements affect periodontitis susceptibility.

Out of 31 orthologous genes underlying the significant QTLs, *Perl*3 and *Perl*4, one genetic locus at *Perl*3 (*CCDC121-GPN1*) was reported to be associated with chronic periodontitis (rs111571364, MAF EUR = 0.02%)(Teumer et al. 2013). However, this association did not replicate in our CP and AgP samples. This could indicate a false positive association of the previous study or be an expression of the trait heterogeneity of periodontitis, as the three GWAS studies applied different diagnostic criteria. The precise function of CCDC121 is unknown and it is not clear if the yet-unknown causative variant has a *cis* effect on *CCDC*121 or on other genes. Interestingly, 3.4 Kb upstream of the CP associated SNP at *CCDC*121 lies a SNP (rs6547741, MAF EUR = 0.50%), which is associated with oral cavity cancer at a genome-wide significance level(Lesseur et al. 2016). The association with an additional trait that affects the oral cavity adds to the hypothesis that this genomic region has a role in oral health. Interestingly, both SNPs are in linkage disequilibrium (D'=1) and for the common variant rs6547741, eQTL studies reported several effects on gene expression on a chromosomal region 200-100kb upstream (from *SNX17*, p= $8.6x10^{-15}$; tissue: skeletal muscle, to *GKCR*, p= $2.3x10^{-8}$; tissue: spleen)(Kristin G et al. 2015), a region that carries numerous GWAS lead SNPS of lipid and glycemic traits, but also Crohn's disease. Taking into account that the mouse QTL encompassed *CCDC*121 but not the genes from *SNPX*17 to *GKCR*, it is possible although speculative that the associated SNPs may exert their *tans*-regulatory effects by influencing *CCDC*121 function.

Although the analysis revealed significant QTL with high resolution in the CC mice, we did not find evidence of association of the orthologous regions in our AgP and CP samples. It is possible that the molecular mechanisms that regulate gene expression are different in humans compared to mice and that the orthologous regions do not carry risk variants in both mice and humans. Accordingly, we may have missed the regions that are relevant for human periodontitis by focusing on the confined mouse QTL. Additionally; the lack of validated associations in the human samples may be explained by the instance that the effects of potential causative variants within the orthologues regions are too small for identification in limited size of our case-control samples. Of note, common GWAS employ sample sizes of up to 100.000s individuals.

By analyzing genes (1,229) in the suggestive QTL, additional five genetic regions suggested to be associated with periodontal disease susceptibility. Two regions *(NRG3, PARK2-PACRG)* showed nominal significant association in the AgP and CP samples, but these association signals were located at different regions of these genes. Interestingly, both genes *PARK2* and *PACRG* are located near the gene plasminogen *(PLG)* which is shown to be significantly associated with AgP (Schaefer et al. 2015) and CP. It is possible that the observed associations at these loci are false positives or that they point to different regulatory elements of the analyzed sub-phenotypes of periodontitis. Two

more loci at *ZNF579* and at *FIZ1-ZNF524* were not associated with our AgP and CP samples, but they had previously been reported to be associated with CP(Teumer et al. 2013). The non-validation of these two reported associations may again indicate false positives or indicate the heterogeneity of the different periodontitis phenotypes.

In conclusion, the CC populations enabled mapping confined QTL that confer susceptibility to alveolar bone loss in mice. However, larger human phenotype-genotype samples and additional genome-wide expression data from gingival tissues are likely required to identify true positive associations.

Acknowledgements

This work was supported by the German Research Foundation DFG (Deutsche Forschungsgemeinschaft; GZ: SCHA 1582/3-1 and SCHA 1582/4-1) and by the Israel Science Foundation [grant number 429/09]. The data for the European-American CP cases were generated by the following research grants of the US-American National Institute of Health (NIH): RO1-DE022527, UL1-TR001111, and RO1-DE021418. The Federal Ministry of Education and Research (01GR0468) supported collection of the popgen control sample. The Dortmund Health Study (DHS) is supported by the German Migraine & Headache Society (DMKG) and by unrestricted grants of equal share from Almirall, Astra Zeneca, Berlin Chemie, Boehringer, Boots Health Care, Glaxo-Smith-Kline, Janssen Cilag, McNeil Pharma, MSD Sharp & Dohme and Pfizer to the University of Muenster (collection of sociodemographic and clinical data). The Institute of Epidemiology and Social Medicine University of Muenster funded Blood collection in the Dortmund Health Study and the Federal Ministry of Research and Education (BMBF, grant no. 01ER0816) supported genotyping. FOCUS was supported by the Federal Ministry of Education and Research BMBF (FKZ 0315540A). The Heinz Nixdorf Foundation (Germany) supported the HNR study. Additionally, the Federal Ministry of Education and Science and the German Research Council (DFG; Project SI 236/8-1, SI 236/9-1, ER 155/6-1) funded it. The German Centre financed Genotyping of the Illumina HumanOmni-1 Quad BeadChips of the HNR subjects for Neurodegenerative Diseaes (DZNE), Bonn. We further thank Corinna Bruckmann, Christof Dörfer, Peter Eickholz, Yvonne Jockel-Schneider, Jörg Meyle, Barbara Noack, and Ingmar Staufenbiel for the recruitment of cases of aggressive periodontitis.

Bibliography:

Berger K (2012). The Dortmund health study. Bundesgesundheitsblatt - Gesundheitsforsch. - Gesundheitsschutz 55:816–821.

Bochenek G, Häsler R, Nour Eddine M, König IR, Loos BG. Jepsen S, Philip R, Schreiber S, Schaefer AS (2013). The large non-coding RNA ANRIL, which is associated with atherosclerosis, periodontitis and several forms of cancer, regulates ADIPOR1, VAMP3 and C11ORF10. Hum. Mol. Genet. 22:4516–4527.

Churchill G, Airey D, Allayee H, Angel JM, Attie A, Beatty J, Beavis W, Belknap J, Bennett B, Berrettini W et al.

(2004). The Collaborative Cross, a community resource for the genetic analysis of complex traits. Nat. Genet. 36:1133–1137.

Corey L a, Nance WE, Hofstede P, Schenkein H a (1993). Self-reported periodontal disease in a Virginia twin population. J. Periodontol. 64:1205–8.

Divaris K, Monda K, North K, Olshan A, Lange E, Moss K, Barros SP, Beck JD, Offenbacher S et al. (2012). Genome-wide Association Study of Periodontal Pathogen Colonization. J. Dent. Res. 91:S21–S28.

Divaris K, Monda K, North K, Olshan A, Reynolds L, Hsueh W, Lange E, Moss K, Barros S, Weyant R et al. (2013). Exploring the genetic basis of chronic periodontitis: A genome-wide association study. Hum. Mol. Genet. 22:2312–2324.

Durrant C, Tayem H, Yalcin B, Cleak J, Goodstadt L, De Villena FP-M, Mott R, Iraqi F (2011). Collaborative Cross mice and their power to map host susceptibility to Aspergillus fumigatus infection. Genome Res. 21:1239–48.

Ernst, F. D.Linden, G. J.; Homuth, G.; Kocher T (2010). Replication of the association of chromosomal region 9p21.3 with generalized aggressive periodontitis (gAgP) using an independent case-control cohort. BMC Med. Genet. 11:119.

Kristin G, David S, Ayellet V, Timothy J, Taylor R, Ellen T, Casandra A, Julian B, Taru T, Monkol L et al (2015). Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. Science. 8;348(6235):648-60.

Freitag-Wolf S, Dommisch H, Graetz C, Jockel-Schneider Y, Harks I, Staufenbiel I, Meyle J, Eickholz P, Noack B, Bruckmann C et al. (2014). Genome-wide exploration identifies sex-specific genetic effects of alleles upstream NPY to increase the risk of severe periodontitis in men. J.Clin. Periodontol. 41:1115–1121.

Genco CA, Cutler CW, Kapczynski D, Maloney K, Arnold RR (1991). A novel mouse model to study the virulence of and host response to Porphyromonas (Bacteroides) gingivalis. Infect. Immun. 59:1255–63.

Hajishengallis G (2014). Periodontitis: from microbial immune subversion to systemic inflammation. Nat. Rev. Immunol. 15:30–44.

Hashim N, Linden G, Ibrahim M, Gismalla B, Lundy F, Hughes FJ, El Karim I (2015). Replication of the association of GLT6D1 with aggressive periodontitis in a Sudanese population. J. Clin. Periodontol. 42:319–324.

Hill C, Gerardo D, James F, Tyroler HA, Chambless LE, Romm J, Disanto A, Barr K, Bergsten J, Conrad J et al. (1989). The atherosclerosis aisk in communities (ARIC) study : design and objectives. Am J Epidemiol. 129(4):687-702.

Howie BN, Donnelly P, Marchini J, Hardy J, Abecasis G (2009). A Flexible and Accurate Genotype Imputation Method for the Next Generation of Genome-Wide Association Studies NJ Schork, editor. PLoS Genet. 5:e1000529.

Iraqi F (2000). Fine mapping of quantitative trait loci using advanced intercross lines of mice and positional cloning of the corresponding genes. Exp. Lung Res. 26:641–649.

Iraqi F, Athamni H, Dorman A, Salymah Y, Tomlinson I, Nashif A, Shusterman A,Weiss E, Houri-Haddad Y, Mott R et al. (2014). Heritability and coefficient of genetic variation analyses of phenotypic traits provide strong basis for high-resolution QTL mapping in the Collaborative Cross mouse genetic reference population. Mamm. Genome 25:109–119.

Iraqi FA, Churchill G, Mott R (2008). The Collaborative Cross, developing a resource for mammalian systems genetics: a status report of the Wellcome Trust cohort. Mamm. Genome 19:379–81.

Lesseur C, Diergaarde B, Olshan AF, Wünsch-Filho V, Ness AR, Liu G, Lacko M, Eluf-Neto J, Franceschi S, Lagiou Pet al. (2016). Genome-wide association analyses identify new susceptibility loci for oral cavity and pharyngeal cancer. Nat. Genet. 48:1544–1550.

Marchini J, Howie BN, Myers S, McVean G, Donnelly P (2007). A new multipoint method for genome-wide association studies by imputation of genotypes. Nat. Genet. 39:906–13.

Mott R, Talbot CJ, Turri MG, Collins AC, Flint J (2000). A method for fine mapping quantitative trait loci in outbred animal stocks. Proc. Natl. Acad. Sci. U. S. A. 97:12649–54.

Müller N, Schulte DM, Türk K, Freitag-Wolf S, Hampe J, Zeuner R, Schröder j, Gouni-Berthold I, Berthold H, Krone W et al. (2015). IL-6 blockade by monoclonal antibodies inhibits apolipoprotein (a) expression and lipoprotein (a) synthesis in humans. J. Lipid Res. 56:1034–42.

Offenbacher S, Divaris K, Barros SP, Moss KL, Marchesan JT, Morelli T, Zhang S, Kim S, Sun L, Beck J D et al. (2016). Genome-wide association study of biologically-informed periodontal complex traits offers novel insights into the genetic basis of periodontal disease. Hum Mol Genet 25:1–17.

Polak D, Wilensky A, Shapira L, Halabi A, Goldstein D, Weiss EI, Houri-Haddad Y (2009). Mouse model of experimental periodontitis induced by Porphyromonas gingivalis/Fusobacterium nucleatum infection: bone loss and host response. J. Clin. Periodontol. 36:406–10.

Rhodin K, Divaris K, North KE, Barros SP, Moss K, Beck JD, Offenbacher, S. (2014). Chronic Periodontitis Genome-wide Association Studies: Gene-centric and Gene Set Enrichment Analyses. J. Dent. Res. 93:882–890.

Schaefer AS, Bochenek G, Jochens A, Ellinghaus D, Dommisch H, Zeldemir A, Akanat E, Graetz C, Harks I, Jockel-Schneider Y et al. (2015). Genetic evidence for PLASMINOGEN as a shared genetic risk factor of coronary artery disease and periodontitis. Circ. Cardiovasc. Genet. 8:159–167.

Schaefer AS, Richter GM, Groessner-Schreiber B, Noack B, Nothnagel M, El Mokhtari NE, Loos BG, Jepsen S, Schreiber S (2009). Identification of a shared genetic susceptibility locus for coronary heart disease and periodontitis. PLoS Genet. 5:1–8.

Schaefer AS, Richter GM, Nothnagel M, Manke T, Dommisch H, Jacobs G, Arlt A, Rosenstiel P, Noack B, Groessner-Schreiber B, et al. (2010). A genome-wide association study identifies GLT6D1 as a susceptibility locus for periodontitis. Hum. Mol. Genet. 19:553–562.

Schmermund A, Möhlenkamp S, Stang A, Grönemeyer D, Seibel R, Hirche H, Mann K, Siffert W, Lauterbach K, Siegrist J et al. (2002). Assessment of clinically silent atherosclerotic disease and established and novel risk factors for predicting myocardial infarction and cardiac death in healthy middle-aged subjects: Rationale and design of the Heinz Nixdorf RECALL study. Am. Heart J. 144:212–218.

Shusterman, Durrant C, Mott R, Polak D, Schaefer A, Weiss EI, Iraqi F, Houri-Haddad Y (2013). Host susceptibility to periodontitis: mapping murine genomic regions. J. Dent. Res. 92:438–43.

Shusterman A, Salyma Y, Nashef A, Soller M, Wilensky A, Mott R, Weiss EI, Houri-Haddad Y, Iraqi F (2013). factor of host susceptibility to periodontitis in the CollaboratiGenotype is an important determinantve Cross and inbred mouse populations. BMC Genet. 14:68.

Sima C, Cheng Q, Rautava J, Levesque C, Sherman P, Glogauer M (2015). Identification of quantitative trait loci influencing inflammation-mediated alveolar bone loss: insights into polygenic inheritance of host-biofilm disequilibria in periodontitis. J. Periodontal Res.

Teumer A, Holtfreter B, Völker U, Petersmann A, Nauck M, Biffar R, Völzke H, Kroemer H, Meisel P, Homuth G et al. (2013). Genome-wide association study of chronic periodontitis in a general German population. J. Clin. Periodontol. 40:977–85.

The genome architecture of the Collaborative Cross mouse genetic reference population. (2012). Genetics 190:389–

401.

Vaithilingam RD, Safii SH, Baharuddin N a., Ng CC, Cheong SC, Bartold PM, Schaefer S, Loos, B. G. (2014). Moving into a new era of periodontal genetic studies: Relevance of large case-control samples using severe phenotypes for genome-wide association studies. J. Periodontal Res.:683–695.

Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, Klemm A, Flicek P, Manolio T, Hindorff T et al. (2014). The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. Nucleic Acids Res. 42:D1001–6.

Van Wijngaarden JP, Dhonukshe-Rutten RAM, van Schoor NM, van der Velde N, Swart KMA, Enneman AW, Van Dijk S, Brouwer-Brolsma E, Zillikens M, Van-Meurs J et al. (2011). Rationale and design of the B-PROOF study, a randomized controlled trial on the effect of supplemental intake of vitamin B12 and folic acid on fracture incidence. BMC Geriatr. 11:80.

Wilensky A, Gabet Y, Yumoto H, Houri-Haddad Y, Shapira L (2005). Three-dimensional quantification of alveolar bone loss in Porphyromonas gingivalis-infected mice using micro-computed tomography. J. Periodontol. 76:1282–6.

Yalcin B, Flint J, Mott R (2005). Using progenitor strain information to identify quantitative trait nucleotides in outbred mice. Genetics 171:673–81.

Yang H, Ding Y, Hutchins LN, Szatkiewicz J, Bell TA, Paigen BJ, Graber J H, De Villena FP-M, Churchill G (2009). A customized and versatile high-density genotyping array for the mouse. Nat. Methods 6:663–6.

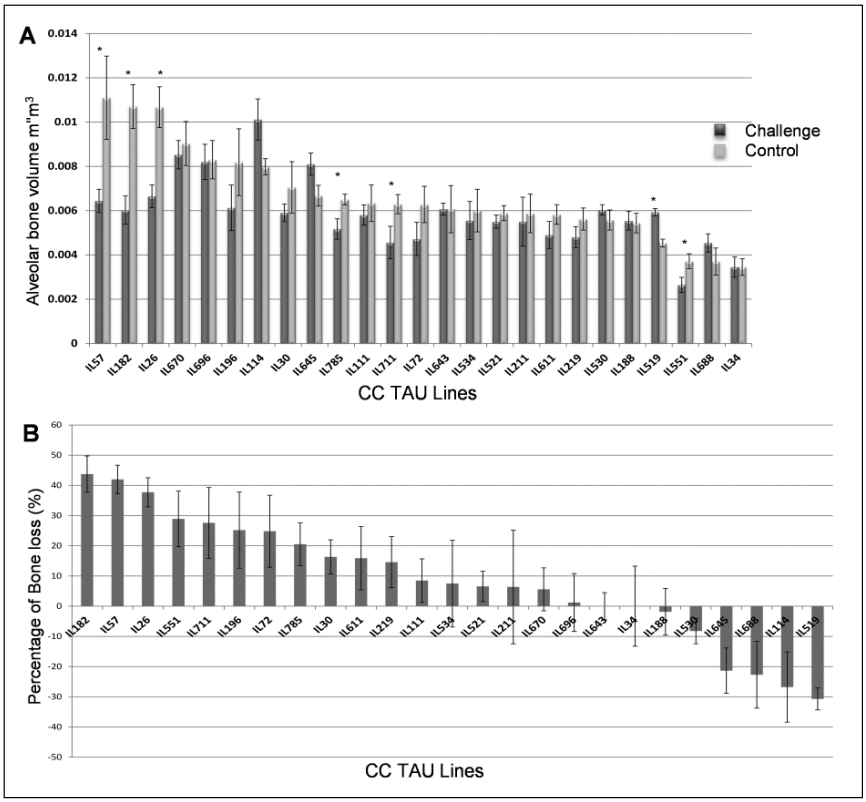

Figure 1. Estimation of alveolar bone phenotypes. (**A**) The means of the alveolar bone volumes (± SEM) of 25 different Collaborative Cross (CC) lines. The x-axis represents the different CC lines while the y-axis represents the mean of the alveolar bone volume in mm^3 evaluated by micro–computed tomography (CT). The black bars represent the mean of the control alveolar bone volume (CBV) while the gray bars represent the mean of residual bone volume after mixed infection (RBV). Asterisks represent the significant differences between the 2 groups ($P < 0.05$). (**B**) Percent alveolar bone volume loss (PBL) due to the mixed infection (± SEM) of 25 different CC lines. The x-axis represents the different CC lines while the y-axis represents the mean percentage of alveolar bone volume loss among the different lines.

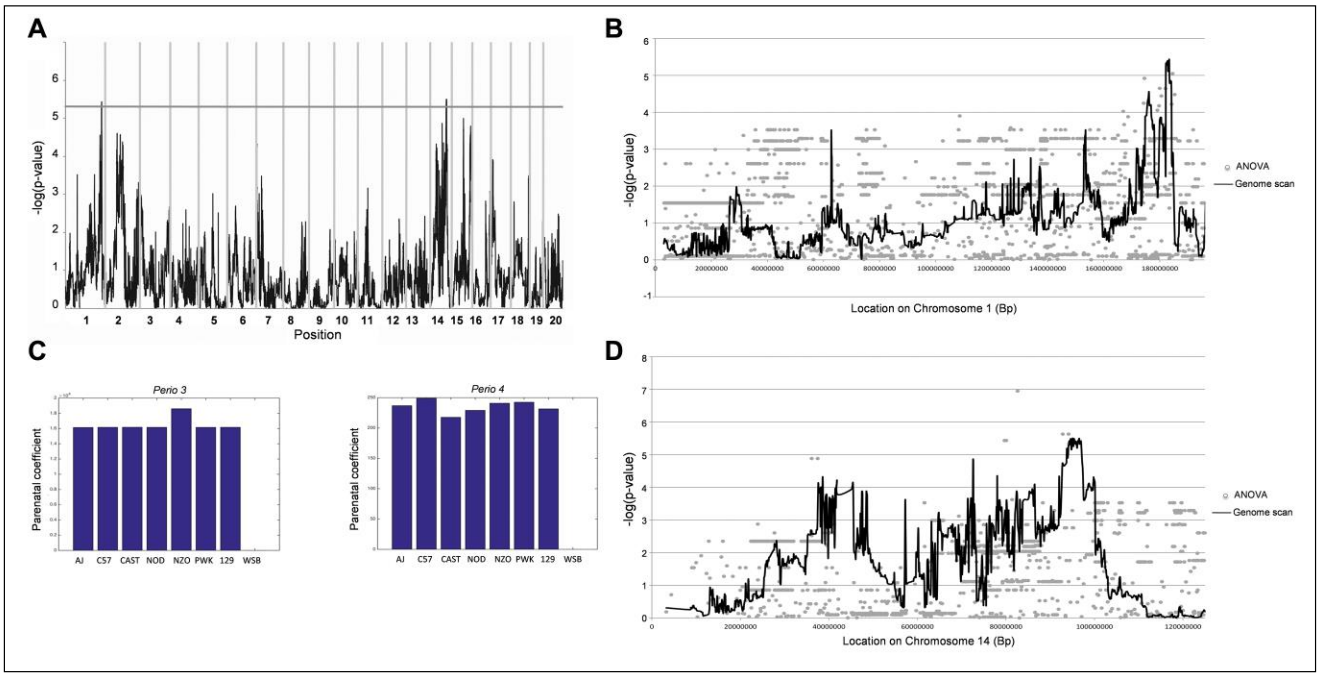

Figure 2. QTL, founder effect and merge analysis. (**A**) Genome scans of susceptibility to alveolar bone volume loss percentage in 25 different Collaborative Cross (CC) lines. The x-axis represents genome location; the y-axis represents the –log *P* of the test of association between locus and percentage of bone loss. Two quantitative trait loci (QTL) associated with percentage of bone loss after infection mapped on chromosome 1 with genome coordinate (181.5 to 182.5 Mbp) and chromosome 14 with genome coordinate (93.5 to 96.5 Mbp). (**B**) Founder effect-estimated haplotype effects at QTL for alveolar bone loss after mixed infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Effects are shown as deviations relative to WSB/EiJ, which is arbitrarily assigned the trait effect of 0. The x-axis of each plot shows the founder strains; the y-axis shows the estimated haplotype effects of the CC founders. A, *Perio*3; B, *Perio*4. Merge analysis of sequence variants at (**C**) *Perio*3 and (**D**) *Perio*4. The x-axis is genome location; y-axis is the –log *P* value of the test of association between locus and alveolar bone loss phenotype. The continuous black lines are sections of the genome scans in A while gray dots are the results of analysis of variance tests of sequence.

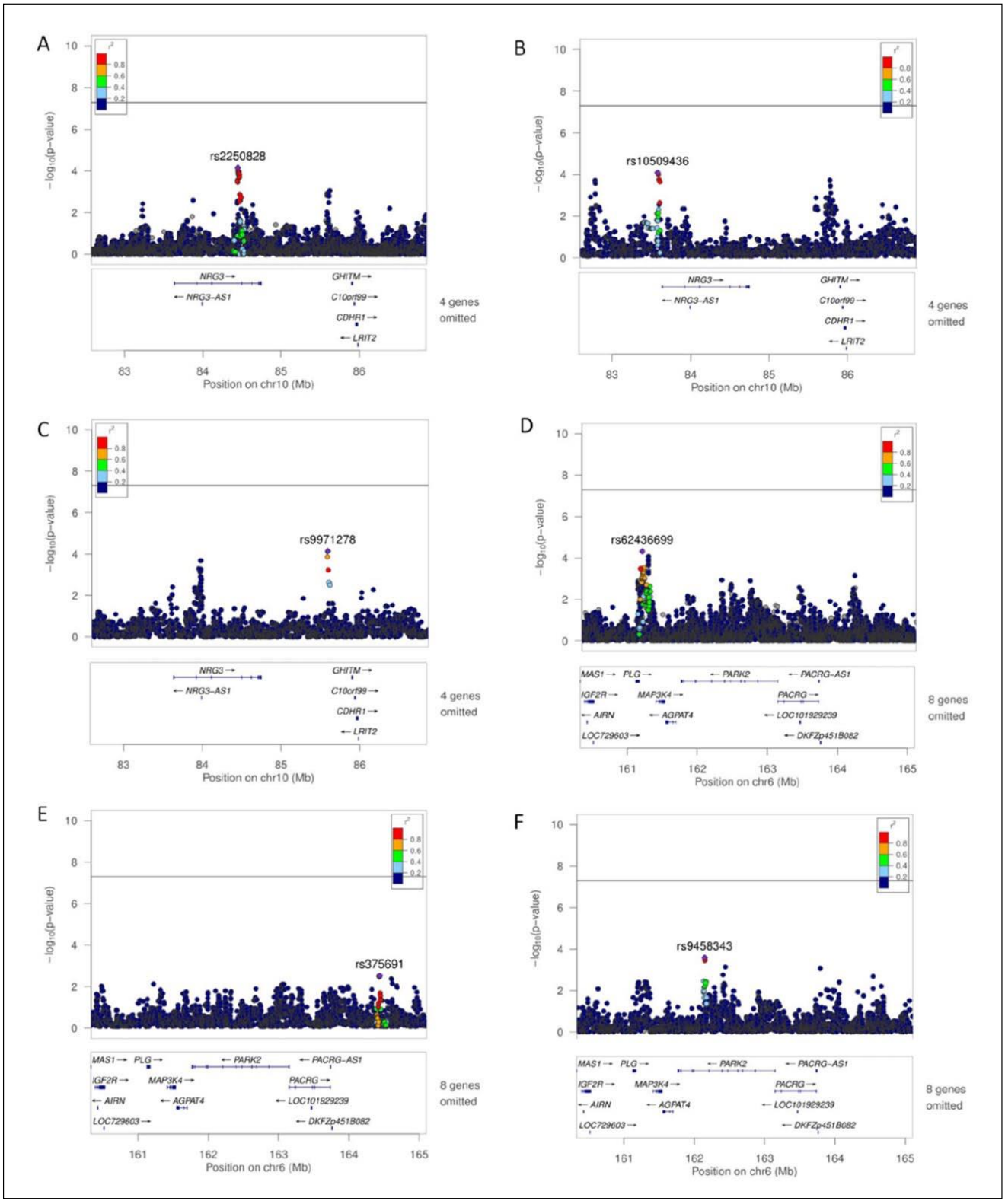

Figure 3. Association plots of imputed genotypes at the NRG3, PARK2, and PACRG loci. Regional association plots of imputed genotypes for aggressive periodontitis (**A**), severe chronic periodontitis (**B**), and moderate chronic periodontitis (**C**) for the chromosomal region spanning NRG3. Regional association plots of imputed genotypes for aggressive periodontitis (**D**), severe chronic periodontitis (**E**), and moderate chronic periodontitis (**F**) for the chromosomal region spanning PARK2 and PACRG. The –log *P* values of the analyzed single-nucleotide polymorphisms (SNPs) were plotted as a function of the genomic SNP position. SNP annotation provided by LocusZoom databases.