# The Novel *ASIC2* Locus Is Associated with Severe Gingival Inflammation

S. Zhang[1,2], K. Divaris[3,4], K. Moss[2], N. Yu[2], S. Barros[1,2], J. Marchesan[1,2], T. Morelli[1], C. Agler[5], S.J. Kim[2], D. Wu[1], K.E. North[4,6], J. Beck[2,7], and S. Offenbacher[1,2]

Abstract: *An increasing body of evidence suggests a significant genetic regulation of inflammatory response mechanisms; however, little is known regarding the genetic determinants of severe gingival inflammation (GI). We conducted a genome-wide association study of severe GI among 4,077 European American adults, participants in the Dental Atherosclerosis Risk in Communities cohort. The severe GI trait was defined dichotomously with the 90th percentile of gingival index ≥2 extent score. Genotyping was performed with the Affymetrix 6.0 array platform, and an imputed set of 2.5 million markers, based on HapMap Phase II CEU build 36, was interrogated. Genetic models were based on logistic regression and controlled for ancestry (10 principal components), sex, age, and examination center. One locus on chromosome 17 met genome-wide statistical significance criteria—lead single-nucleotide polymorphism: rs11652874 (minor allele frequency = 0.06, intronic to* ASIC2 *[acid-sensing ionic channel 2, formerly named* ACCN1*]; odds ratio = 2.1, 95% confidence interval = 1.6 to 2.7,* P = $3.9 \times 10^{-8}$*). This association persisted among subjects with severe periodontitis and was robust to adjustment for microbial plaque index. Moreover, the minor (G) allele was associated with higher levels of severe GI in stratified analyses among subsets of participants with high load of either "red" or "orange" complex pathogens, although this association was not statistically significant. While these results will require replication in independent samples and confirmation by mechanistic studies, this locus appears as a promising candidate for severe GI. Our findings suggest that genetic variation in* ASIC2 *is significantly associated with severe GI and that the association is plaque independent.*

Knowledge Transfer Statement: *Persistent gingival inflammation reflected by bleeding usually precedes ongoing attachment loss or periodontal disease progression. Our findings suggest that genetic variation in* ASIC2 *that is associated with severe gingival inflammation might be used as a genetic marker to identify people at higher risk for periodontal disease. Ongoing studies to uncover the mechanistic link between* ASIC2 *and gingival inflammation could lead to novel therapeutic interventions.*

Keywords: periodontal disease(s)/periodontitis, gingivitis, genetics, genomics, plaque/plaque biofilms, bacteria

## Introduction

Periodontal disease is an abnormal inflammatory response to the pathogenic bacteria present in the biofilm. Gingival inflammation (GI), as determined by bleeding upon probing, is a clinical hallmark for both gingivitis and periodontitis. Gingivitis affects >80% of Americans, while 47% of American adults have periodontitis (Eke et al. 2012). In addition to stress

DOI: 10.1177/2380084416645290. [1]Department of Periodontology, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [2]Center for Oral and Systemic Diseases, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [3]Department of Pediatric Dentistry, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [4]Department of Epidemiology, UNC Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [5]Center for Oral and Craniofacial Health Sciences, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [6]Carolina Center for Genome Sciences, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [7]Department of Dental Ecology, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. Corresponding author: S. Offenbacher, 3501F Koury Oral Health Sciences Building, 385 S. Columbia Street, Chapel Hill, NC 27599-7455, USA. Email: Steven_Offenbacher@unc.edu

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exerted by the local microbial burden, it is now well-established that host genomics contributes to periodontal disease susceptibility. Twin studies by Michalowicz et al. (2000) suggested that heritability accounts for about 50% of the total phenotypic variability of periodontal disease. More recently, in a study of >10,000 Swedish twin pairs, Mucci et al. (2005) reported heritability estimates of 39% and 33% for women and men, respectively. In a more recent report, our group used a genome-wide association (GWA) approach to show that approximately 22% of phenotypic variance observed in severe chronic periodontitis can be explained by a GWA study (GWAS) set of approximately 650,000 genotyped single-nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) ≥5% (Divaris et al. 2013).

Similar to other complex trait diseases, predisposition to periodontal disease is likely polygenic (Kinane et al. 2005). Most previous studies interrogating the genetic role in periodontal disease were based on a candidate-gene approach (Kornman et al. 1997; Tervonen et al. 2007; Kobayashi et al. 2009; Laine et al. 2012). GWASs provide an unbiased approach for generating hypotheses on nonrare genetic variants for common diseases, such as periodontitis. By the end of 2013, approximately 14,000 SNPs had reported GWAs with >600 traits (Welter et al. 2014). Although SNPs identified by GWAS may explain a small proportion of the observed variance for most diseases (Gibson 2010), this methodology has brought about notable successes in the study of obesity and metabolic traits (Locke et al. 2015). There is promise that similar advancements will eventually become possible in oral and periodontal research.

There are only a few GWASs on chronic periodontitis (Divaris et al. 2013; Teumer et al. 2013; Feng et al. 2014; Shaffer et al. 2014; Shimizu et al. 2015). While no significant genome-wide SNP hits have been identified so far, several suggestive loci have been reported. Divaris and colleagues (2013) reported the *NIN* locus as showing suggestive evidence of association with severe periodontitis. This locus was highlighted in a separate GWAS of periodontal disease by Shaffer et al. (2014), although the top SNP in the latter study was approximately 350 kb upstream of *NIN* and not in linkage disequilibrium with the lead SNP reported by Divaris et al. In addition, the *NPY* locus, which also emerged from the Atherosclerosis Risk in Communities (ARIC) study report, was subsequently highlighted by Freitag-Wolf et al. (2014) as being associated with periodontitis among men (albeit tagging SNPs were not in linkage disequilibrium), as well as by a gene-centric reanalysis of ARIC reported by Rhodin et al. (2014). Even fewer GWASs have explored loci associated with periodontal phenotypes defined by quantitative clinical signs rather than disease categories. Two notable exceptions include a study by Shaffer et al. (2014) that used probing depth ≥5.5 mm present in at least 2 sextants as the outcome for their GWA analyses and a study by Divaris et al. (2012) that reported several suggestive genetic loci associated with high bacterial pathogen colonization by "red" complex (*Porphyromonas gingivalis, Tannerella Forsythia*, and *Treponema denticola*), "orange" complex (*Prevotella intermedia, Fusobacterium nucleatum, Prevotella nigrescens*, etc.), and *Aggregatibacter actinomycetemcomitans* among European American participants of the ARIC cohort.

There is compelling evidence that persistent GI reflected by bleeding upon probing combined with residual probing depth after periodontal treatment has prognostic value for ongoing attachment loss or disease progression (Lang et al. 1986; Claffey et al. 1990; Matuliene et al. 2008). GI may also contribute to a systemic hyperinflammatory state that is etiologically related to other chronic diseases and pathologies (Scannapieco 2004). We do know that the microbial biofilm is etiologically associated with gingivitis (Loe et al. 1965). Yet, it is also known that inflammation, including the production of cytokines, is highly genetically regulated (de Craen et al. 2005). However, no systematic effort has been undertaken to study the genetic underpinning of GI as an independent clinical sign with a GWA approach. It is also unknown whether a genetic association, if present, is modified by the level of exposure to periodontal pathogens. We hypothesize that genetic variants that interact with known periodontal pathogens are associated with the severe GI trait as reflected by bleeding upon probing. To test this hypothesis, we used the well-defined Dental ARIC cohort and conducted the first GWA scan for genetic risk loci associated with extensive severe GI and examined these associations among subgroups with varying levels of subgingival periodontal pathogens and categorical classifications of chronic periodontitis.

## Materials and Methods

### Study Population and Measurements

We used data from the previously reported GWAS performed in a subset of European American subjects who were participants in the ARIC study (ARIC Investigators 1989). The ARIC cohort study aimed to study atherosclerosis, cardiovascular disease risk factors, and outcomes and originally recruited 15,792 community-dwelling residents in 4 U.S. communities between 1987 and 1989. As an ancillary study, Dental ARIC recruited a subset of ARIC participants in the fourth ARIC visit (1996-1998). During their dental visit, 6,017 dentate ARIC subjects had a complete full-mouth dental examination, which included measurements of probing depth, attachment loss, and bleeding upon probing at 6 sites per tooth, including third molars, number of missing teeth, and gingival index (Loe and Silness, 1963). Plaque index (PI) was recorded for each tooth according to the method introduced by Silness and Loe (1964). Subgingival plaque samples were also collected from 973 European American Dental ARIC participants. For the present analyses, we included only European

American participants and excluded individuals whose genotyping did not meet quality control criteria. Therefore, in this report we used genotype and clinical data from a subset of 4,077 Dental ARIC subjects.

### Genotyping, Quality Control, and Imputation

Detailed description of genotyping and data processing were described elsewhere (Divaris et al. 2012; Divaris et al. 2013). Briefly, DNA was extracted from blood samples. Genotyping was performed with the Affymetrix Genome-Wide Human SNP Array 6.0 chip containing 906,600 SNP markers. After quality control, an imputation to 2.5 million SNP markers was performed with 669,450 high-quality genotyped SNPs and the MACH program (version 1.0.16) based on HapMap Phase II CEU build 36. Further SNP exclusion included the following criteria: imputation quality score <0.8, missing data rate >10% after imputation, and MAF <5%.

### Quantification of Periodontal Organisms in Plaque Samples by DNA Checkerboard

Levels of bacteria within plaque samples were determined as previously described (Socransky et al. 1994). One plaque sample taken from the subgingival mesiobuccal site of the maxillary right first molar was used from each subject and hybridized to a DNA chromosomal checkerboard for the 8 periodontal pathogens present in the red or orange complexes defined by Socransky et al. (1998). Organism levels were expressed as counts based on known microbial standards. A "high" load was defined as those in the upper quartile (25%) range of counts, and a "low" load, those in the lowest three-quartiles count range. Eight periodontal pathogens were included for analyses: *Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola, Tannerella forsythia* (formerly *Bacteroides forsythus*), *Campylobacter rectus, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), and *Prevotella nigrescens*.

### Analytic Strategy

The clinical trait of interest was the extent of moderate to severe GI as reflected by the ordinal gingival index, wherein "0" reflects normal gingiva; "1," mild inflammation but no bleeding upon running probe in the sulcus; "2," moderate inflammation with bleeding upon running probe in the sulcus; and "3," severe inflammation with a tendency for spontaneous bleeding. Extent scores were calculated as the percentage of sites exhibiting gingival index ≥2 (EGIGE2) among all examined sites for each participant. The severe GI trait was defined dichotomously as gingival index extent score in the 90th percentile (top 10%), whereas the continuous GI variable was used for exploratory and stratified analyses. Genetic models were based on logistic regression, adjusting for ancestry (10 principal components), age, sex, and examination center. A conventional genome-wide significance level of $P < 5 \times 10^{-8}$ was used for single-marker discovery in the GWAS, whereas loci with suggestive evidence of association ($P < 5 \times 10^{-6}$) are also reported. All genetic analyses were performed with the ProbABEL software. We utilized Manhattan and LocusZoom plots (Pruim et al. 2010) to visualize the genetic loci of interest. We followed HUGO Gene Nomenclature to report genes. Exploratory analyses of the continuous GI trait, as well as analyses stratified by chronic periodontitis diagnosis and high versus low pathogen load, were based on generalized linear models, adjusting for ancestry, age, sex, examination center, and microbial plaque levels and accounting for multiple comparisons. We followed the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) checklist guidelines for the reporting of findings.

## Results

### Single-Marker GWAS

Genomic inflation for the severe GI association analysis was low ($\lambda = 1.008$). The quantile-quantile plot (Appendix Fig.) did not reveal any evidence of residual population stratification. The full list of GWAS results, including estimates of association for all 2.5 million SNPs, is available at http://genomewide.net/public/aric/dental/gingivitis/EGIGE2.txt. One locus on chromosome 17q11.2 met genome-wide statistical significance criteria (Fig. 1A) for the severe GI trait. The lead SNP in this locus was rs11652874: MAF (G) = 0.06, odds ratio (OR) = 2.1, 95% confidence interval = 1.6 to 2.7, $P = 3.9 \times 10^{-8}$. This imputed SNP has an imputation quality score of 0.998, which is considered excellent. This SNP is intronic to the *ASIC2* gene (acid-sensing ionic channel 2; previously named *ACCN1*; Fig. 1B), within the intron region between exons 2 and 3, and in tight linkage disequilibrium with 9 additional intronic SNPs (Fig. 1C). We found no association of this SNP with clinical diagnoses of chronic periodontitis (e.g., 4-level case definition per the Centers for Disease Control and Prevention / American Academy of Periodontology [CDC/AAP]; $P = 0.14$; Table). We found 3 additional loci with suggestive evidence of association ($P < 5 \times 10^{-6}$) with severe GI: *PHGDH* (lead SNP rs894049, $P = 1.2 \times 10^{-6}$, OR = 1.62, MAF [G] = 0.14), *NRXN1* (lead SNP rs1520455, OR = 1.47, $P = 4.0 \times 10^{-6}$, MAF [C] = 0.37), and *ACVR1* (lead SNP rs7578180, OR = 1.43, $P = 4.5 \times 10^{-6}$, MAF [C] = 0.33). *ASIC2* encodes a member of the degenerin/epithelial sodium channel superfamily and is likely involved in neurotransmission (Krishtal 2003).

### Stratified Analyses

To further explore the *ASIC2* locus signal, we examined the lead SNP's association with the continuous GI trait (EGIGE2) stratified by the 4-level CDC/AAP chronic periodontitis diagnoses (Fig. 2). Not surprisingly, in both *ASIC2* genotype groups, EGIGE2 was higher among participants with more advanced (severe) disease status versus those with milder disease. We found no association between rs11652874 and EGIGE2 among

**Figure 1.** rs11652874 in the *ASIC2* gene locus is associated with severe gingival inflammation. (**A**) Manhattan plot of genome-wide association analysis results of severe gingival inflammation (binary trait based on EGIGE2q90) in the Atherosclerosis Risk in Communities cohort ($n$ = 4,077). (**B**) Locus zoom visualization of the 17q11.2 locus (*ASIC2*, lead SNP: rs11652874) flanking ±400 kb. The vertical axis represents the $\log_{10}$-transformed $P$ value for each SNP. A schematic representation of the *ASIC2* gene exons in relation to the lead SNP (rs11652874) is shown in the inset. (**C**) Locus zoom visualization of the 17q11.2 locus flanking a zoomed-in ±25-kb region. SNP, single-nucleotide polymorphism.

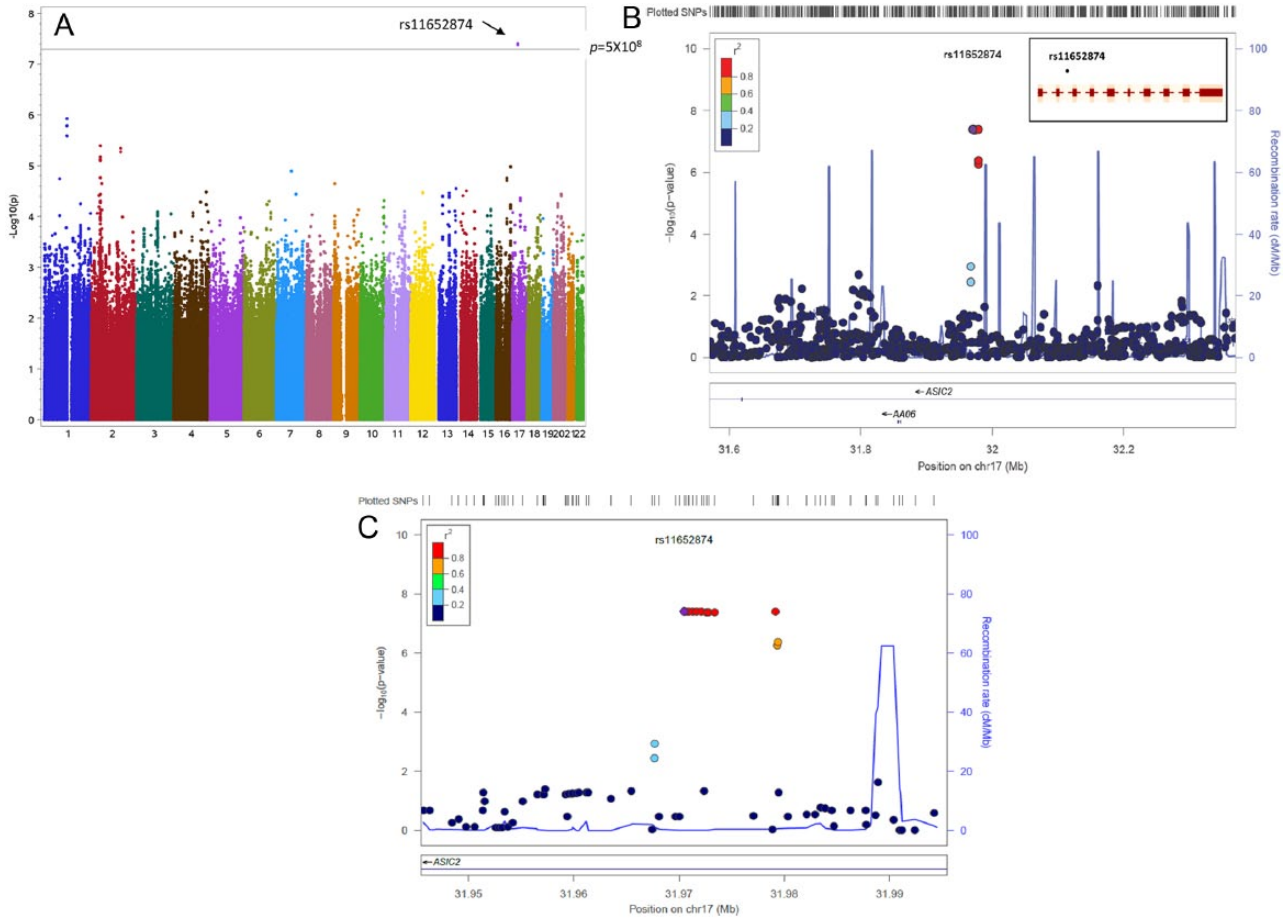


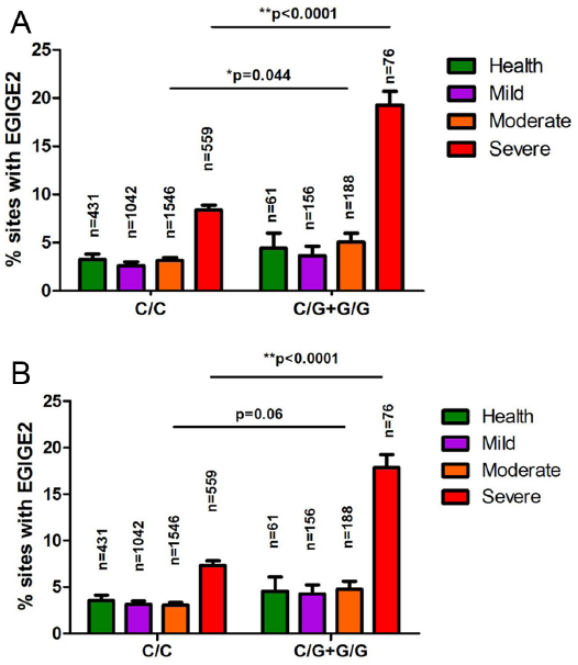

**Table.**
Genotype Distribution of rs11652874 according to Periodontal Disease Diagnoses.

| Genotype | Periodontal Disease Definition, *n* (%)[a] | | | | |
|---|---|---|---|---|---|
| | Healthy | Mild | Moderate | Severe | Total |
| C/C | 492 (10.3; 11.7) | 1,178 (24.7; 28.0) | 1,817 (38.1; 43.2) | 720 (15.1; 17.1) | 4,207 (88.3; 100.0) |
| C/G+G/G[b] | 67 (1.4; 12.0) | 177 (3.7; 31.7) | 214 (4.5; 38.3) | 101 (2.1; 12.3) | 559 (11.7; 100.0) |
| Total | 559 (11.7) | 1,355 (28.5) | 2,031 (42.6) | 821 (17.2) | 4,766 (100.0) |

Four-level case definition based on the American Academy of Periodontology and Centers for Disease Control and Prevention (Eke et al. 2012).
[a]For cells that have 2 values in parentheses, the first value denotes the percentage among the entire sample, and the second, the percentage among subjects with the same genotype.
[b]$P$ = 0.14 ($\chi^2$ test).

subjects with periodontal health ($P$ = 0.67) or mild periodontitis ($P$ = 0.13). However, among subjects with moderate or severe periodontitis, the EGIGE2 is significantly higher among rs11652874 (G) allele carriers versus noncarriers ($P$ = 0.04 for moderate periodontitis and $P$ < 0.0001 for severe periodontitis) after adjustment for study design features,

**Figure 2.** Association of rs11652874 polymorphisms (minor allele [G] carriers vs. noncarriers) with gingival inflammation extent score ≥2 (EGIGE2) stratified by chronic periodontitis diagnosis (case status classification per the American Academy of Periodontology and Centers for Disease Control and Prevention), adjusting for (**A**) age, sex, ancestry (10 principal components), and examination center and (**B**) microbial plaque levels, age, sex, ancestry (10 principal components), and examination center.




participant characteristics, and ancestry (Fig. 2A). This association was somewhat attenuated after further adjustment for plaque score (Fig. 2B) in the moderate periodontitis stratum (unadjusted: beta = 1.92, $P = 0.04$, vs. adjusted: beta = 1.69, $P = 0.06$), whereas it remained significant ($P < 0.0001$) in the severe periodontitis stratum. In other words, the increased GI among those with the *ASIC2* minor (G) allele polymorphism was not substantially affected by microbial plaque levels, particularly among participants with severe chronic periodontitis.

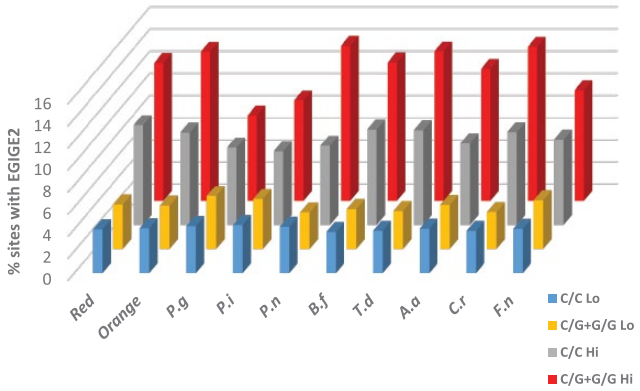
To further explore the possible effect modification by microbial plaque, next we examined possible SNP × periodontal pathogen interactions that might account for the higher gingival index score. Therefore, we examined whether rs11652874 (G) allele carrier status modified the gingival inflammatory response to bacteria of the red and the orange complexes, as well as individual bacterial species. High periodontal pathogen loads were associated with statistically increased gingival index scores as compared with lower bacterial loads, independent of rs1652874 genotype (Fig. 3; *P* values not shown). Although a trend for an association between rs1652874 (G) allele and periodontal pathogen levels was noted, we found no statistically significant association after adjustment for multiple comparisons.

## Discussion

In this genome-wide investigation conducted among the well-defined Dental ARIC cohort, we found a novel locus associated with severe GI. The identified polymorphism is intronic to the *ASIC2* gene and not associated with CDC/AAP chronic periodontitis diagnosis. The rs11652874 minor (G) allele was associated with the most severe gingival bleeding among those with severe chronic periodontitis, and this was independent of microbial plaque levels. We tested the hypothesis that levels of specific periodontal microorganisms may alter the clinical response according to the *ASIC2* polymorphism. Although some trends were noted, the extent of GI was not associated with this polymorphism within red or orange pathogen high- and low-load groups. These findings suggest that there may be additional loci or biological or environmental/behavioral risk factors that are specific to severe disease and interact to produce extremely high gingival index scores (Manolio et al. 2009).

The lead SNP in the *ASCI2* locus is located in the gene's second intron of the gene. Acid-sensing ion channels play an important role in the nervous system (Lingueglia and Lazdunski 2015). We found no important functional annotation or expression quantitative trait loci information about this SNP in publicly available databases such as SNPnexus (http://snp-nexus.org/) and GTEx (http://www.gtexportal.org/). *ASIC2* has been reported to be ubiquitously expressed in the mammalian peripheral and central nervous system. Residing in the free sensory nerve endings that are in contact with local tissue, this molecule may regulate the physiologic response of microvessels to fluctuations of local oxygen consumption. During inflammation, the drop of pH due to increased oxygen utilization leads to *ASIC2* activation and the release of vasoactive substances from the other branches of nerve endings is triggered (Krishtal 2003). The involvement of *ASIC2* in inflammation is supported by experiments with *ASIC2* knockout mice, in which several inflammatory markers, such as TGF-β (transforming growth factor-β) and TAPA-1 (target of antiproliferative antibody 1), were all upregulated when compared with wild-type controls (Gannon et al. 2015). Although there is evidence supporting a plausible role for *ASIC2* contributing to gingival hyperinflammatory phenotype, further replication and mechanistic studies are warranted for its validation.

**Figure 3.** Comparison of severe gingival inflammation extent scores (EGIGE2) by levels of 8 periodontal pathogens in the red and orange complexes as determined by DNA checkerboard; bacterial loads were dichotomized into high level (the top quartile) and low level (lowest 3 quartiles) and stratified by genotypes at rs11652874. *A.a, Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*); *B.f, Tannerella forsythia* (formerly *Bacteriodes forsythus*); *C.r, Campylobacter rectus; F.n, Fusobacterium nucleatum; P.g, Porphorymonas gingivalis; P.i, Prevotella intermedia; P.n, Prevotella nigrescens; T.d, Treponema denticola*.




The neurotransmitter and nervous system signaling pathways have been repeatedly reported in our GWASs and others, as well as in our transcriptome studies (Offenbacher et al. 2009; Divaris et al. 2013). The exact role of neuropathways in periodontal health and disease remains to be elucidated; nevertheless, neuropeptides are known to regulate acid-sensing ion channels, as detailed by Vick and Askwith (2015). Ingenuity pathway analyses based on data from our experimental transcriptome gingivitis study indicate that there are 4 genes upstream to *ASIC2* that are upregulated during gingivitis induction (*ASIC2* was not present on our transcriptome array for scanning), suggesting the activation of inflammatory pathways involving *ASIC2*. These genes are *NRXN1, CHD8, CTNNB1*, and *NLGN1* (Offenbacher et al. 2009). Noteworthy, several chemokine genes, including *CCL2, CCL8, CCL11, CCL7*, and *CCL13*, are located downstream of *ASCI2* on chromosome 17q12. The involvement of those CCL chemokines in periodontal inflammation is well documented (Silva et al. 2007). The signal in the *ASIC2* locus may also highlight other possibly associated genes in nearby genome regions, and these chemokine genes would serve as excellent additional candidates.

In addition to *ASIC2*, we identified several suggestive loci close to genes with known functions. For example, rs894079 is located in the 5′ untranslated region of *PHGDH*, which encodes the enzyme involved in the early steps of L-serine synthesis in animal cells (Zogg 2014). Rs1520455 is intronic to *NRXN1*, which encodes neurexin, a presynaptic protein diffusely distributed in neurons and a key component in neuronal network (Bottos et al. 2011). Rs7578180 is intronic to *ACVR1*, which encodes a receptor for activins, which are dimeric growth and differentiation factors belonging to the TGF-β superfamily of signaling proteins (Goumans et al. 2003). Although those SNPs did not meet criteria for genome-wide statistical significance, they appear as promising candidates for future studies.

Our study is limited by the absence of a replication sample to externally validate the observed association between rs11652874 and GI. Although our results are derived from a moderately sized GWAS, including >4,000 community-dwelling participants in a well-defined cohort, an independent replication of this SNP and other polymorphisms highlighted in this study is warranted. We acknowledge that independent replication and mechanistic validation are crucial because this association—although statistically robust—may simply represent a false-positive finding (Ioannidis 2005). Moreover, we considered levels of only 8 key pathogens within the red and orange complexes and cannot exclude that other organisms unique to the moderate and severe disease states might be critical and interact with this polymorphism to produce high levels of GI. For example, we and others have reported on the importance of organisms such as *Fretibacterium* and *Treponema* species in periodontal disease (Marchesan et al. 2015). This recent Human Microbiome Identification Microarray study identified 272 subgingival microbial species and defined *Synergistetes*- and *Spirochaetes*-dominated microbial community structures that were significantly associated with increased probing depth and bleeding upon probing (Marchesan et al. 2015). Those pathogens and many other important organisms were not included in analyses presented here, which were limited to the key "classical" cultivable pathogens.

In summary, in these GWA analyses of severe GI among the Dental ARIC European American population we found a genome-wide significant association of a chromosome 17q12 locus, marked by a SNP intronic to the *ASIC2* gene. The association persisted among groups with severe periodontitis and was independent of clinical levels of microbial plaque; neither loads of specific red or orange complex pathogens modified this association. These results provide support for the conduct of further studies in this or nearby genome regions to explore if and how common genetic variation in *ASIC2* plays a role in severe GI.

## Author Contributions

S. Zhang, K. Divaris, contributed to conception, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; K. Moss,

contributed to data acquisition and analysis, drafted the manuscript; N. Yu, contributed to data acquisition and interpretation, drafted and critically revised the manuscript; S. Barros, T. Morelli, S.J. Kim, D. Wu, contributed to data interpretation, critically revised the manuscript; J. Marchesan, contributed to data acquisition and analysis, critically revised the manuscript; C. Agler, contributed to data analysis, drafted the manuscript; K.E. North, contributed to data analysis and interpretation, drafted and critically revised the manuscript; J. Beck, contributed to conception, critically revised the manuscript; S. Offenbacher, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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