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Genetic Variants Associated With Development of TMD and Its Intermediate Phenotypes: The Genetic Architecture of TMD in the OPPERA Prospective Cohort Study

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Abstract: **Genetic risk factors are believed to combine with environmental exposures and contribute to the risk of developing temporomandibular disorder (TMD). In this prospective cohort study, 2,737 people without TMD were assessed for common genetic variation in 358 genes known to contribute to nociceptive pathways, inflammation, and affective distress. During a median follow-up period of 2.8 years, 260 people developed first-onset TMD. Hazard ratios were computed as measures of association between 2,924 single-nucleotide polymorphisms and TMD incidence. After correction for multiple testing, no single single-nucleotide polymorphism was significantly associated with risk of onset TMD. However, several single-nucleotide polymorphisms exceeded Bonferroni correction for multiple comparison or false discovery rate thresholds (.05, .1, or .2) for association with intermediate phenotypes shown to be predictive of TMD onset. Nonspecific orofacial symptoms were associated with voltage-gated sodium channel, type I, alpha subunit (*SCN1A*, rs6432860, $P = 2.77 \times 10^{-5}$) and angiotensin I–converting enzyme 2 (*ACE2*, rs1514280, $P = 4.86 \times 10^{-5}$); global psychological symptoms with prostaglandin-endoperoxide synthase 1 (*PTGS1*, rs3842803, $P = 2.79 \times 10^{-6}$); stress and negative affectivity with amyloid-β (A4) precursor protein (*APP*, rs466448, $P = 4.29 \times 10^{-5}$); and heat pain temporal summation with multiple PDZ domain protein (*MPDZ*, rs10809907, $P = 3.05 \times 10^{-5}$). The use of intermediate phenotypes for complex pain diseases revealed new genetic pathways influencing risk of TMD.**

***Perspective:** This article reports the findings of a large candidate gene association study of first-onset TMD and related intermediate phenotypes in the OPPERA Study. Although no genetic markers predicted TMD onset, several genetic risk factors for clinical, psychological, and sensory phenotypes associated with TMD onset were observed.*

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Idiopathic pain disorders such as temporomandibular disorder (TMD) are heterogeneous in presentation and multifactorial in etiology,[25] both of which may be due in part to genetic influences. We have previously hypothesized that persistent pain conditions result from 2 parallel domains of vulnerability, each composed of a temporally dependent mosaic of subclinical traits.[17] The first domain, pain amplification, may arise because of injury/trauma, proinflammatory physiological state, impaired pain regulatory mechanisms, or autonomic dysregulation. The second domain, psychological distress, can manifest as somatization, anxiety, depression, stress response, or catastrophizing. Each of these domains is modulated by numerous biological processes and probably influenced by many genes.

Genetic risk factors likely play a role in the etiology of TMD, based on a twin study that attributed 44% of variation in the occurrence of TMD to genetic inheritance.[74] Case-control studies of TMD have reported associations with genes in the serotonergic pathway, including serotonin transporter *SLC6A4*,[36,54] serotonin 2A receptor *HTR2A*,[50] and the serotonin metabolic enzyme tryptophan hydroxylase 1 *TPH*.[22] Adrenergic mechanisms have also been implicated, based on findings that polymorphisms in both the β2-adrenergic receptor *ADRB2*[16] and catechol-*O*-methyltransferase *(COMT)*[15] are associated with TMD risk. The high proportion of female TMD subjects in many studies has prompted consideration of the estrogen receptor gene *ESR1* as a strong candidate for association with TMD, although with conflicting results.[38,41,43,60] Similarly, commonly occurring genetic variants have been associated with fibromyalgia and chronic widespread pain, including *ADRB2*,[37,76] *COMT*,[6,47,72] *TAAR1*,[67] *HTR2A*,[48] and *SLC6A4*.[10,52] Other painful conditions found to have genetic associations include headache,[65] irritable bowel syndrome,[62] vulvar vestibulitis,[35] and low back pain.[12,13,51,69]

In the first phase of the Orofacial Pain: Prospective Evaluation and Risk Assessment (OPPERA) study, we assessed 358 candidate pain genes in a case-control study of 348 chronic TMD subjects and 1,612 TMD-free controls.[66] No single-nucleotide polymorphisms (SNPs) were statistically significantly associated with chronic TMD after correction for multiple testing, but findings supported a contribution from candidate genes including *HTR2A*, *COMT*, glucocorticoid receptor (*NR3C1*), calcium/calmodulin-dependent protein kinase type IV (*CAMK4*), muscarinic cholinergic receptor (*CHRM2*), interferon-related developmental regulator 1 (*IFRD1*), and the G protein–coupled receptor kinase 5 (*GRK5*).

An alternative strategy for discovering genetic contributions to TMD is to investigate genetic influences on intermediate phenotypes that contribute to TMD. The contribution of genetic influences relative to environmental factors may be stronger for intermediate phenotypes. Four criteria have been proposed for this approach[28]: the intermediate phenotype 1) is associated with TMD in the population, 2) is heritable, 3) manifests in an individual independent of active disease, and 4) cosegregates with TMD in families. Other papers in this issue address the first criterion by identifying phenotypes that predicted risk of developing first-onset TMD. The prospective cohort fulfills the third criterion because each phenotype was assessed at enrollment, prior to development of TMD.

Here we present findings from the OPPERA prospective cohort study, in which people without TMD at enrollment were followed for up to 5 years. One aim was to identify genetic polymorphisms that predicted incidence of first-onset TMD. Twenty-three genes belonging to major neurotransmitter systems were chosen as primary candidates; the remaining set of 335 genes was considered a discovery panel. A second aim was to identify SNPs that were associated with intermediate phenotypes that predicted TMD incidence.

Methods

Study Setting and Participants

The details of recruitment and characterization of the OPPERA prospective cohort have been described fully elsewhere[4] and are summarized here. A total of 3,263 initially pain-free subjects were recruited by advertisements, e-mails, flyers, and word of mouth between May 2006 and November 2008. They were recruited from communities in and around academic health centers at 4 U.S. study sites: Baltimore, MD; Buffalo, NY; Chapel Hill, NC; and Gainesville, FL. These subjects included both males and females of diverse racial and ethnic backgrounds, and were aged 18 to 44 years at enrollment. Only subjects (n = 2,737) who returned at least 1 follow-up screening questionnaire were included in the prospective study of first-onset TMD. For this subset of OPPERA subjects, the mean age at enrollment was 27.1 years (standard deviation = 7.8 years); 1,630 were female and 1,107 were male (female-male ratio = 1.5:1). Approximately half (n = 1,612) of this prospective cohort sample was used previously in the OPPERA baseline case-control study of chronic TMD.[66] These subjects, enrolled without TMD at baseline, were considered controls who were contrasted with 185 subjects who had examiner-verified chronic TMD (ie, "cases"). None of the chronic TMD cases from that study were used in any analysis described in the present prospective study of first-onset TMD.

Ethical Conduct of Research With Humans

The OPPERA study was reviewed and approved by institutional review boards at each of the 4 study sites

and at the data coordinating center, Battelle Memorial Institute. Participants verbally agreed to a screening interview done by telephone and provided signed, informed consent for all other study procedures, including blood draw and genetic analysis.

Genotyping

This article focuses on genetic variants assessed at baseline that have been described in detail elsewhere[66] and summarized here.

At enrollment, whole blood was collected by venipuncture from study participants who provided consent for genotyping. Blood was collected into 5-mL ethylenediamine tetra-acetic acid (EDTA)–containing polyethylene tubes (Vacutainer; Beckton, Dickinson and Company, Franklin Lakes, NJ), which were stored at −80°C. Genomic DNA was purified utilizing protocols based on Qiagen Extraction Kits from Cogenics, Inc (now Integrated Laboratory Systems, Morrisville, NC).

Samples were genotyped using the Algynomics (Chapel Hill, NC) Pain Research Panel,[67] a dedicated chip-based platform utilizing the Affymetrix MegAllele technology. The panel assesses 3,295 SNPs representing 358 genes known to be involved in systems relevant to pain perception (complete list provided through http://www.algynomics.com/pain-research-panel.html). The panel includes genes putatively involved in intermediate processes underlying TMD, such as nociceptive transmission, inflammation, and mood and affect. The SNPs selected for the panel prioritize functional variants such as those that code for nonsynonymous changes or result in differences in expression or alternative splicing, whereas others were selected as representative markers of regions with high linkage disequilibrium, containing many correlated SNPs that are inherited in blocks, in order to "tag" untyped variation.

Twenty-three genes (Supplementary Table 1) were chosen a priori as "first tier" candidate genes, and as described previously,[66] the probability of type I error was adjusted to account for tests of their 211 SNPs in the analysis reported below. Quality assessment of Pain Research Panel genotypes was performed using PLINK v.1.07 (Broad Institute, Cambridge, MA)[58] as previously described.[66] An identity-by-state analysis was performed using principal components analysis (PCA) on the genotypes to cluster individuals according to racial heritage. The first 2 principal components (eigenvectors) were retained for use as covariates representing racial background in the association testing, in order to adjust for population stratification.[57] Genotyping results were returned for 3,221 unique samples, which included enrollees in the prospective cohort study as well as cases for the case-control study. The overall genotyping call rate was 99.1%, and repeated sample concordance was 99.8%.

Phenotypic Assessment

At 3-month intervals after enrollment, study participants were asked to complete a screening questionnaire that asked about TMD pain symptoms.[4] Those reporting symptoms were invited to study clinics for a follow-up examination that determined presence or absence of painful TMD using OPPERA's implementation of the research diagnostic criteria for TMD.[19] Specifically, the 260 incident cases satisfied 2 criteria for TMD: 1) symptoms of orofacial pain reported for ≥5 days/month and 2) examiner findings of TMD myalgia, arthralgia, or both. For descriptive purposes, the rate of first-onset TMD was calculated as the number of people with first-onset TMD divided by sum of follow-up intervals.

A large number of potential risk factors for onset TMD were evaluated in OPPERA, including measures related to clinical symptoms, psychosocial profile, somatic sensitivity, and autonomic response. Given the large number of raw and derived variables assessed in OPPERA subjects at enrollment, we narrowed our selection of phenotypes for genetic analysis to reduce the burden of multiple testing. For measures of psychological status and experimental pain sensitivity, we used PCA to recalculate factor scores first described in our baseline case-control study.[24,29] This reduced the large number of raw variables measured in each domain, thereby mitigating the number of tests requiring Bonferroni adjustment and producing more stable measures of the underlying construct compared to individual measures. The PCA findings for this prospective cohort study are described elsewhere in this issue.[26,30] Furthermore, we designated as intermediate phenotypes only those measures associated with first-onset TMD in our initially TMD-free subjects, following the definition of Gottesman et al.[28] Using these criteria, we selected 8 characteristics as intermediate phenotypes for this analysis, listed here with their respective univariate effect on the incidence rate of TMD (hazard ratio [HR] adjusted for site and demographic variables). Dependent variables for the intermediate phenotypes taken from the clinical measures[53,63] were 1) the number of comorbid health conditions (0, 1, or ≥2, HR = 1.39 for 1 and HR = 2.87 for ≥2 comorbid health conditions), 2) the number of nonspecific orofacial symptoms (0, 1 to 2, or ≥3, HR = 1.98 for 1 or 2 symptoms and HR = 2.89 for ≥3 symptoms), and 3) global score of the Pittsburg Sleep Quality Index[9] (a continuous variable, HR = 1.4). Tenderness at 10 separate masticatory muscle groups (temporalis, masseter, posterior mandibular and submandibular, lateral pterygoid area, and temporomandibular joint, both right and left sides) during examination procedures was also associated with first-onset TMD. For the intermediate phenotype analysis, we created a single summary variable by counting 4) the number of tender masticatory muscles for each individual, which was itself a strong predictor of TMD incidence (continuous variable, HR = 1.33). Intermediate phenotypes derived from the battery of psychosocial measures[26] included 5) the principal component from factor analysis of psychosocial measures that signified global psychological symptoms (continuous variable, HR = 1.37) and 6) the principal component from factor analysis of psychosocial measures signifying stress and negative affectivity (continuous variable, HR = 1.17). Intermediate phenotypes associated with incident TMD from among the quantitative sensory testing (QST) variables[30] were 7) the

principal component from factor analysis of QST measures signifying pressure pain thresholds (a continuous measure, HR = 1.14) and 8) the principal component from factor analysis of QST measures signifying heat pain temporal summation (but only in the lowest tertile of first-pulse responders; see the accompanying paper in this issue[30] for details; continuous variable, HR = 1.54).

Association With TMD Incidence and Intermediate Phenotypes

To test hypotheses about associations between genetic variants and TMD incidence rate in the 2,737 subjects with follow-up data, univariate HRs were computed using Cox proportional hazard regression as implemented in the R package GenABEL v. 1.5-1.[3] HRs with 95% confidence intervals (CIs), assuming codominant effects of genotype, were computed with adjustment for study site and the first 2 racial eigenvectors. Alternative genetic models, including tests of dominant and recessive inheritance, were also tested. To evaluate stratum-specific genetic effects, additional tests were performed as above in males and females separately and in whites and African Americans separately (without adjustment by racial eigenvectors). For the intermediate phenotypes, all subjects enrolled without TMD at baseline were included in analyses (n = 3,037 with phenotype and genotype data). Association tests assuming codominant effects were performed by fitting a generalized linear model in R, in which the quantitative or binary observed phenotype was the dependent variable. Effect size and direction were estimated using beta coefficients for quantitative traits, and odds ratios (ORs) for binary traits and stratum-specific tests were performed as above.

Because we tested a large number of SNPs and several intermediate phenotypes, it was necessary to adjust our standard of statistical significance in order to maintain a desired experiment-wide $\alpha = .05$. It was also important to weigh the risk of false positives against the risk of false negative results (type II errors), which are common in association studies that use conservative adjustments (such as a Bonferroni correction) evaluating large numbers of correlated markers with small effect sizes. We used the false discovery rate (FDR) method[8] to control the expected proportion of false positives among associations reported as significant, adopting a threshold FDR of .2. This method balances the considerations of retaining power to detect true associations after correction for multiple testing, while acknowledging that some results considered significant will be false positives.

Results

TMD Incidence

The cohort of 2,737 initially TMD-free people was followed for a total of 7,404 person-years (median = 2.8 years per person), during which time 260 people developed first-onset TMD, yielding an annual incidence rate of 3.5% of people per annum. Additional details regarding the demographics of subjects followed in the prospective cohort and those with acute TMD are provided in the methods paper in this issue.[4] The quantile-quantile (Q-Q) plot (Fig 1) of the distribution of observed $-\log(P$ values) from tests of association with TMD incidence did not differ markedly from the null distribution (genomic control $\lambda < 1$), suggesting that adjusting for covariates was adequate to minimize the effects of population stratification and other potential sources of systematic bias.[57]

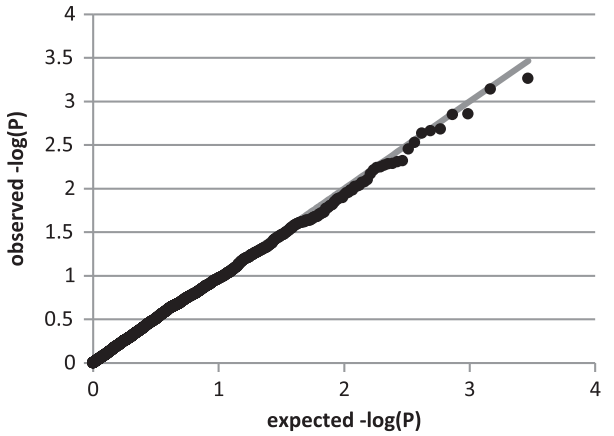

Figure 1. Q-Q plot of all SNPs in the test of genetic association with TMD incidence. Black dots represent the observed $-\log_{10}(P$ value) for all SNPs passing quality control criteria, compared with the expected values under the null distribution (gray diagonal line).

The results of the top 20 SNPs in the full set of SNPs are provided in Table 1. As indicated by the Q-Q plot, no statistically significant associations were observed. There were also no SNPs with a Bonferroni-corrected statistically significant association for TMD incidence among Tier 1 genes (see Supplementary Table 2 for top SNPs, and Supplementary Fig 1 for Q-Q plot).

Intermediate Phenotype Association Results

We next assessed the association between the SNP panel and intermediate phenotypes predictive of TMD incidence. As described elsewhere in this issue,[26,30,53,63] a number of measures relevant to domains of hypersensitivity and psychological distress were evaluated prospectively for association with first-onset TMD, and those that were predictive of TMD incidence were identified as intermediate phenotypes. These included 4 clinical variables[53,63] (the number of comorbid health conditions, the number of nonspecific orofacial symptoms, the number of masticatory muscle groups painful during examination procedures, and the global score of the Pittsburg Sleep Quality Index); 2 factors derived from the PCA of psychosocial measures[26] (global psychological symptoms and stress and negative affectivity); and 2 factors from the PCA of QST variables[30] (pressure pain threshold and heat pain temporal summation).

Results for tests of association between each SNP and each intermediate phenotype are depicted in Q-Q plots (Fig 2; Supplementary Fig 2 shows additional plots for tests with no results with FDR <.2 and tested nonintermediate phenotypes; Supplementary Fig 3 shows Manhattan plots

Table 1. Top Association Test Results for TMD Incidence: OPPERA Prospective Cohort Study, 2006–2011

				Mode of Inheritance						Sex				Race			
				Additive		Dominant		Recessive		Female		Male		White		Black	
SNP	Gene	Chr	Minor Allele	HR	P	HR	P	HR	P	HR	P	HR	P	HR	P	HR	P
rs12415832	*VEGF*	6	C	.70	.00055	.62	.00035	.68	.087	.79	.064	.56	.0018	.78	.054	.60	.0085
rs1563826	*ERBB2*	17	G	1.38	.00072	1.57	.0019	1.47	.025	1.39	.0059	1.34	.071	1.37	.0092	1.35	.076
rs1076292	*EPHB2*	1	C	.73	.0014	.73	.027	.55	.0025	.75	.020	.70	.034	.73	.0078	.73	.11
rs2072100	*CCL5*	17	A	1.47	.0014	1.57	.0012	1.48	.31	1.49	.0075	1.42	.092	1.27	.16	1.76	.0034
rs728273	*ERBB2*	17	A	1.35	.0021	1.53	.0023	1.38	.10	1.35	.015	1.33	.079	1.31	.030	1.41	.045
rs3782221	*MC4R*	18	C	1.70	.0022	1.63	.014	4.43	.0014	1.66	.021	1.71	.055	1.37	.35	1.59	.027
rs2367707	*DRD2*	11	G	1.37	.0023	1.25	.13	1.88	.00032	1.43	.0060	1.28	.15	1.45	.0040	1.33	.14
rs1448239	*INADL*	1	G	1.33	.0030	1.39	.022	1.54	.0098	1.32	.020	1.32	.084	1.18	.16	1.49	.025
rs255097	*ERBB2*	17	C	1.32	.0035	1.54	.0025	1.30	.16	1.32	.021	1.32	.088	1.32	.026	1.31	.11
rs6967334	*HIF1A*	14	T	.69	.0048	.65	.016	.62	.055	.65	.013	.78	.21	.68	.070	.68	.032
rs3804452	*INADL*	1	C	.72	.0049	.68	.0068	.59	.11	.76	.066	.66	.035	.77	.056	.64	.10
rs3782202	*ITGAM*	16	T	.72	.0052	.67	.0060	.64	.12	.75	.039	.68	.071	.81	.11	.61	.18
rs4883544	*DRD2*	11	C	1.32	.0052	1.34	.034	1.62	.011	1.38	.011	1.22	.21	1.50	.0018	1.25	.17
rs1550798	*SCN2A*	2	G	.72	.0054	.69	.0073	.61	.13	.70	.014	.77	.18	.71	.021	.76	.18
rs7800170	*P2RY6*	11	T	.68	.0057	.69	.017	.31	.044	.66	.018	.72	.13	.73	.074	.64	.049
rs7687621	*ITGAM*	16	T	.73	.0058	.68	.0070	.64	.12	.74	.030	.72	.12	.80	.098	.65	.19
rs2363561	*GRIN2B*	12	C	.74	.0062	.71	.012	.62	.079	.76	.052	.71	.063	.67	.0051	.93	.72
rs3787535	*CCL5*	17	C	1.38	.0068	1.46	.0069	1.46	.28	1.41	.022	1.36	.12	1.18	.35	1.63	.0066
rs1557545	*CRHR2*	7	A	.76	.0079	.70	.023	.71	.046	.78	.041	.75	.090	.76	.019	.58	.049
rs6685551	*INADL*	1	T	2.24	.0084	2.27	.017	6.23	.071	3.78	.00043	.90	.87	NA	NA	2.21	.011

Abbreviation: Chr, chromosome.

NOTE. Multivariable proportional hazards models of time-to-event for first-onset TMD, with recruitment site and race as covariates. HRs and *P* values (P) are provided for the top 20 SNPs, ranked by *P* value, in the test assuming an additive model of inheritance. Additional columns are provided indicating association test results for these SNPs by alternative model of inheritance (dominant or recessive); stratified by sex, using an additive model of inheritance; and stratified by race, using an additive model of inheritance and without adjustment for race.

for all tested phenotypes). The results for Q-Q plots are presented with FDR = .05, .1, and .2 thresholds assessing deviation from the null distribution. Effect size estimates (relative to the minor allele) and *P* values for all results that exceeded the FDR = .02 threshold are provided in Table 2, for the full cohort and by strata.

Association tests were performed for 4 clinical intermediate phenotypes[53,63]: the number of 1) comorbid health conditions, 2) nonspecific orofacial symptoms, 3) and masticatory muscle groups painful during examination procedures and 4) the global score of the Pittsburg Sleep Quality Index. The strongest association was with nonspecific orofacial symptoms, including jaw stiffness, cramping, fatigue, pressure, soreness, and ache.[53] Three linked SNPs within the voltage-gated sodium channel, type I, alpha subunit gene *SCN1A* approached the corrected significance threshold for association, including a nonsynonymous Ala>Thr polymorphism (best SNP rs6432860, minor allele [MA] = A, $P = 2.77 \times 10^{-5}$, OR = 1.42, 95% CI = 1.21–1.68). Another strong association was observed at an intronic SNP in the angiotensin I–converting enzyme 2 gene, *ACE2* (rs1514280, MA = T, $P = 4.86 \times 10^{-5}$, OR = 1.32, 95% CI = 1.15–1.51). No associations were observed beyond the FDR = .2 threshold for the other clinical measures.

Two psychological factors associated with incident TMD were tested for genetic association: global psychological symptoms, which includes characteristics such as somatization, anxiety, depression, hostility, and psychoticism; and stress and negative affectivity, which represents measures of state and trait anxiety, perceived stress, and neuroticism.[26] The strongest association with global psychological symptoms, exceeding Bonferroni-corrected significance, was a synonymous Pro-Pro SNP in the prostaglandin-endoperoxide synthase 1 gene *PTGS1* (rs3842803, MA = C, $P = 2.79 \times 10^{-6}$, beta = −.22, 95% CI = −.31 to −.13). This SNP is rare in whites (minor allele frequency [MAF] < .01) but fairly common in African Americans (MAF = .24); the SNP remains strongly associated in African Americans alone ($P = 3.45 \times 10^{-4}$). One polymorphism was associated with the stress and negative affectivity factor, a promoter region SNP near the amyloid precursor protein gene, *APP* (rs466448, MA = A, $P = 4.29 \times 10^{-5}$, beta = .11, 95% CI = .06–.17).

The QST variables were summarized by 5 principal components, of which only 2 were examined as intermediate phenotypes.[30] One SNP was associated with heat pain temporal summation in the lowest tertile of first-pulse responders,[30] located in an intron of the multiple PDZ domain protein *MPDZ* (rs10809907, MA = C, $P = 3.05 \times 10^{-5}$, beta = .16, 95% CI = .09–.24). No SNPs were significantly associated with the pressure pain threshold principal component.

Discussion

In this prospective study, we did not observe significant variation in TMD incidence according to genetic

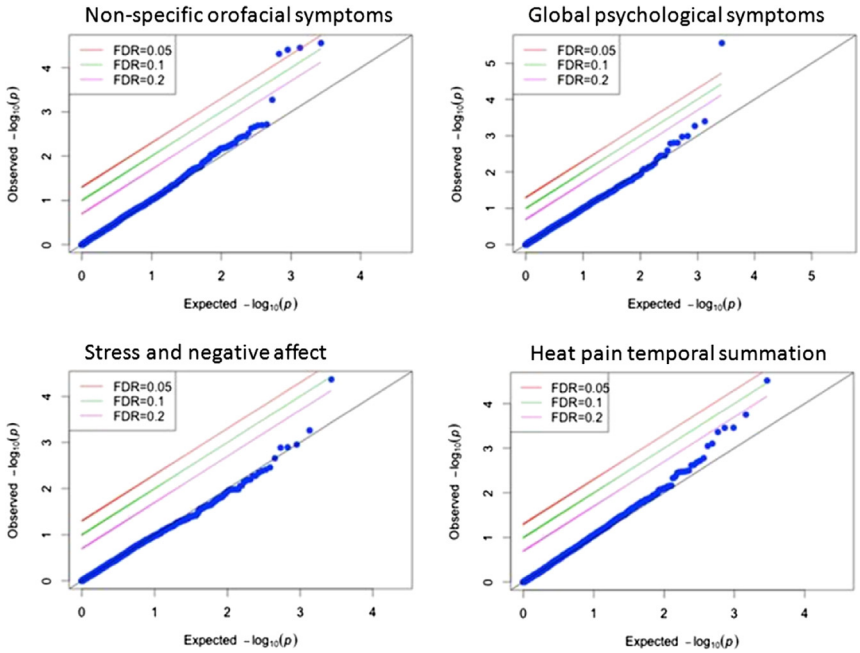

Figure 2. Q-Q plots for intermediate phenotypes with SNPs associated at FDR <.2. Q-Q plots of all SNPs in 4 tests of genetic association with intermediate phenotypes of first-onset TMD. Blue dots represent the observed $-\log_{10}(P$ value) for all SNPs passing quality control criteria, compared with the expected values under the null distribution (gray diagonal line). Additional colored lines are displayed above the gray line, indicating the thresholds for the FDR as indicated in the key.

polymorphisms from the panel of candidate genes. Although the cohort was large, the relatively small number of people who developed TMD limited our power to detect genetic effects of small size. Despite nominating a small "first-tier" panel of putative genes, and statistical methods that guarded against type II error, the lack of observed associations may have occurred for several reasons. Although a genetic basis for chronic TMD has been established, acute-onset TMD may be determined in large part by environmental factors, such as trauma, infection, or stress, and only a subset of acute TMD cases will become chronic.[20] Likewise, the causes of first-onset TMD may be more heterogeneous than for chronic TMD. If so, genetic variants may be more readily discovered by clustering cases of first-onset TMD into groups with similar pathophysiology, and therefore hypothetically similar genetic determinants. Replication of suggestively associated SNPs in a second independent cohort, which this study lacks, would be necessary to provide additional evidence of genetic determination of first-onset TMD. To further explore the role of common genomic variation in TMD, the OPPERA group has continued enrollment of healthy and TMD subjects in preparation for a well-powered genome-wide association study (GWAS).

In contrast to the lack of results for first-onset TMD, we observed evidence for genetic association in a number of pronociceptive intermediate phenotypes. The objective was to find SNPs associated with intermediate phenotypes in our population limited to initially TMD-free individuals, in order to find genetic loci that increase the risk of first-onset TMD. We therefore did not use random sampling of the overall population, which narrows the generalizability of these findings to people without TMD. The SNPs associated with intermediate phenotypes possibly contribute to TMD incidence by increasing the risk of developing these intermediate phenotypes. The implicated genes were derived from a pool of pain candidate genes, each with a known physiological mechanism supporting its relevance to development of TMD. However, there is still much yet to be understood regarding

Table 2. Association Results Significant at FDR = .2: OPPERA Prospective Cohort Study, 2006–2011

Phenotype	*SNP*	*Gene*	*Minor Allele*	*MAF White*	*MAF Black*	*Effect Size*	*SE*	P *Value*
Nonspecific orofacial symptoms	rs6432860	*SCN1A*	A	.31	.18	1.43*	.11	2.77E–05
Nonspecific orofacial symptoms	rs1461193	*SCN1A*	G	.31	.18	1.42*	.11	3.55E–05
Nonspecific orofacial symptoms	rs2298771	*SCN1A*	C	.31	.18	1.42*	.11	3.93E–05
Nonspecific orofacial symptoms	rs1514280	*ACE2*	A	.34	.21	1.32*	.08	4.86E–05
Global psychological symptoms	rs3842803	*PTGS1*	C	<.01	.24	−.22	.09	2.79E–06
Stress and negative affect	rs466448	*APP*	A	.51	.13	.11	.05	4.29E–05
Heat pain temporal summation	rs10809907	*MPDZ*	C	.31	.76	.16	.04	3.05E–05

Abbreviations: MAF, minor allele frequency (minor allele = effect allele); SE, standard error of the effect size.
NOTE. Effect size is expressed as OR for binary phenotypes (nonspecific orofacial symptoms) and designated by an asterisk (*), and as the beta coefficient for continuous phenotypes (factor scores).

the etiologic pathway from genetic risk marker to pronociceptive domain through to TMD onset.

Two genes, *SCN1A* and *ACE2*, were associated with the clinical measure of nonpainful orofacial symptoms, one of the predictors of TMD incidence. *SCN1A* encodes the alpha subunit of the voltage-gated sodium channel $Na_v1.1$, which is involved in the generation and propagation of action potentials in sensory nerves. Mutations in *SCN1A* have been reported to cause several types of epilepsy, especially those associated with febrile seizures,[75] and the gene has also been associated with short-term memory performance in a GWAS.[55] Rare mutations of *SCN1A* have been implicated in hereditary migraine,[18] although the degree to which common variation in the gene affects normal sensation is unknown. Notably, this gene belongs to the same family as *SCN9A*, which encodes the $Na_v1.7$ channel and which has been implicated in mendelian disorders of spontaneous pain and insensitivity as well as altered pain thresholds and risk of chronic pain disease.[59] Tight linkage disequilibrium across the *SCN1A* locus resulted in several panel SNPs displaying evidence of association in our study. The strongest-associated SNP was rs6432860, a synonymous Val752Val substitution in exon 13; another associated SNP (rs2298771) was a nonsynonymous amino acid substitution in exon 16 (rs2298771, Thr1056Ala) of unknown functional significance. Determining the true effect variant responsible for differential perception of nonpainful orofacial symptoms is challenging, as numerous polymorphisms in tight linkage disequilibrium within *SCN1A* result in alterations in function or expression of the $Na_v1.1$ channel.[55]

The second gene associated with nonpainful orofacial symptoms was *ACE2*, which codes for the angiotensin I–converting enzyme 2. In accordance with the function of the renin-angiotensin system in regulating blood pressure and fluid balance, numerous studies have attributed genetic differences in risk of hypertension to variation in *ACE2*,[45] although no studies have reported association with any pain or sensory symptoms. Angiotensin-related peptides have been posited as neurotransmitters in the periaqueductal gray[56] and other pain-relevant brain areas, where they modulate pro- and antinociceptive pathways.[11,46] In addition to angiotensin I, endogenous peptide substrates of ACE include bradykinin, substance P, and opioids such as dynorphin and enkephalin,[21,73] suggesting that the angiotensin system plays a pivotal role underlying the relationship between blood pressure and pain sensitivity. Pharmacologic inhibition of ACE has been associated with increase in nociceptive thresholds and tolerance[31] and risk of complex regional pain syndrome,[14] suggesting that lower levels of ACE activity due to genetic variation might underlie altered somatic sensitivity. The *ACE2* locus is located on the X chromosome, and the effect of this SNP was stronger in males (OR = 1.35) than females (OR = 1.27). The SNP observed in this study is located in an intron between exons 14 and 15, and is therefore likely to be a tag of a nearby ungenotyped polymorphism of unknown function.

An SNP in the *PTGS1* gene was associated with the psychosocial factor representing global psychological symptoms, a significant and strong predictor of TMD incidence (HR = 1.35), and a discriminant of chronic TMD cases from controls. This gene encodes prostaglandin-endoperoxide synthase 1, also known as COX-1, which catalyzes the production of prostaglandins and is therefore pivotal in the regulation of neuronal sensitivity to pain and the mediation of the inflammatory response. Polymorphisms in this gene result in resistance to aspirin,[33] a COX inhibitor, and have been associated with risk of colorectal cancer[71] and functional dyspepsia[1]; no genetic differences in pain sensitivity or risk of painful disease have been reported. Prostaglandins are involved in an array of physiological processes, suggesting that genetic factors that influence baseline or stimulated levels of prostaglandins could alter somatic sensitivity and awareness of autonomic activity, nociception, vascular muscle constriction or dilation, and gastrointestinal function. The SNP associated with global psychological symptoms, rs3842803, is located in the terminal exon 11 of the gene, where it results in a Pro503Pro synonymous change. The effect of this SNP was a decrease in the global psychological symptoms in African Americans carrying the minor allele, indicating that the genetic effect is likely to inhibit prostaglandin production by attenuating the activity of COX-1.

A second association with a psychosocial factor was observed between the stress and negative affectivity factor and an SNP of the *APP* gene. Expressed by neurons, the amyloid-β precursor protein (APP) is involved in synapse formation and neuronal plasticity; although its function is not yet fully understood, the expression of APP has been proposed as a neuroprotective response to stress.[64] Proteolysis of APP results in β-amyloid, a major component of plaques in the brain that have been associated with the development of Alzheimer disease. Risk of Alzheimer disease, especially its familial or early-onset variant, has been associated with polymorphisms of *APP*.[2,32] Cognitive ability and cognitive aging may also be genetically modulated by the gene,[34] suggesting that *APP* polymorphism may underlie deficits in the capacity to handle stressful life events in affected individuals. The associated SNP, rs466448 (−1023 T/C), is found in the promoter region of the gene and has been shown to increase expression levels of the protein.[42] The effect allele was associated with increased stress factor scores in the OPPERA cohort, suggesting a genetic link between higher levels of APP and higher perception of stress.[61,64,70]

A single gene, *MPDZ*, was associated with a QST phenotype predictive of TMD onset, heat pain temporal summation. This gene encodes a multiple PDZ domain protein, also known as MUPP1, that functions as a scaffolding protein for several G protein–coupled receptors involved in nociception and analgesia, including serotonergic[7] and GABAergic[5] receptors. Polymorphisms in *MPDZ* have been associated with alcoholism, both in mice[23] and in humans.[39] It has been suggested that *MPDZ* variation acts on alcohol dependence via the *N*-methyl-D-aspartate–dependent

α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid trafficking cascade,[40] which as a regulator of glutamate-related excitatory neurotransmission would be highly relevant to the phenotype of temporal summation of thermal pain as well. The SNP associated with heat pain temporal summation was located in an intron near exon 28 of the gene and does not have any known functional significance.

Although this study provides evidence for several genes contributing to the etiology of TMD, it has a number of limitations that impact the interpretation of the findings. The power to detect clinically meaningful HRs for TMD was low. Power was generally high for quantitative traits but was still dependent on variables such as allele frequency and the underlying effect size of the SNPs. The proportion of trait variance due to genetic factors is poorly understood for TMD and the intermediate phenotypes, especially in a heterogeneous sample such as OPPERA with many environmental sources of variance. The use of derived variables such as factor scores, though intended to mitigate the problem of multiple testing, makes it difficult to interpret differences in outcome measures due to genetic factors. It is also possible that our selection of candidate genes based on known relevance to pain pathways did not include the primary genes that contribute to risk of first-onset TMD. Precedence for this comes from the large proportion of replicable associations discovered by GWASs that are not found in or near loci of known function or relevance to disease.[27] Another caveat to the interpretation of this study is that we cannot generalize genetic association results for intermediate phenotypes to the general population, as the study design did not include subjects with orofacial pain and was not a true cross section of the population. Finally, a statistical association between a gene and a trait of interest does not fully explain the biological basis for that relationship; further studies are required to characterize the functional consequences of these genetic polymorphisms that result in alterations in nociceptive and psychological traits.

These genes demonstrating association should be examined in other cohorts, as the power in this study alone was limited and meta-analytic methods have succeeded in confirming numerous true genetic associations with effect sizes too small to detect in single low-powered studies.[44,49,68,77] These genetic variants will be considered candidates to test association in cohorts of acute and chronic TMD of more substantial statistical power that are currently being collected. Furthermore, despite the large number of genes and variants tested, we are unable to cover the full extent of genetic variability that may influence TMD risk, such as rare variants, or genes not currently known to be relevant to pain. Instead, we have endeavored to test genes already implicated in pain processing, using a candidate gene analysis. Although this minimized the amount of multiple testing needed to assess the observed risk factors, it will be important to extend these findings in other studies using GWAS and other high-dimension methods. This study has shown the utility of considering intermediate phenotypes, which are likely to be more strongly genetically determined and can be measured in the full cohort to improve power. By dissecting the genetic architecture underlying the physiological and behavioral domains of pain susceptibility, novel etiologic pathways and therapeutic approaches may be revealed.

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Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpain.2013.09.004.

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Supplementary Appendix

Elaboration of Methods Used in Smith et al

The Orofacial Pain Prospective Evaluation and Risk Assessment (OPPERA) study is a prospective cohort study designed to investigate the etiology of first-onset temporomandibular disorder (TMD). Institutional review boards at each study site approved study procedures, and participants provided signed, informed consent. Full details of enrollment, follow-up, and statistical analyses are provided elsewhere in this issue (see[1]) and are summarized here.

Recruitment, Eligibility Criteria, and Enrollment

Between May 2006 and November 2008, potential study participants were recruited using advertisements, e-mails, and flyers at 4 U.S. study sites: Baltimore, MD; Buffalo, NY; Chapel Hill, NC; and Gainesville, FL. Eligibility criteria were age 18 to 44 years, good health, no history of facial injury or surgery, no significant symptoms of TMD pain, no previous diagnosis of TMD, and an absence of TMD myalgia and TMD arthralgia on clinical examination. On enrollment, participants completed a telephone interview and self-administered questionnaires assessing hypothesized risk factors for TMD. During a 3-hour clinical visit, autonomic function was monitored and quantitative sensory tests measured sensitivity to painful stimuli. Study examiners recorded clinical characteristics of muscles and joints of the head, neck, and body and they verified absence of TMD.

Genotyping

At each OPPERA site, whole blood was collected by venipuncture from study participants who provided consent for genotyping. Blood was collected into 5-mL ethylenediaminetetraacetic acid–containing polyethylene Vacutainers (Vacutainer, Beckton, Dickinson and Company), which were stored at −80°C. Each sample was labeled with a unique, bar-coded identifier label. Genomic DNA was purified using protocols based on Qiagen extraction kits at Cogenics, Inc (now Integrated Laboratory Systems Inc, Morrisville, NC).

Samples were genotyped using the Algynomics (Chapel Hill, NC) Pain Research Panel, a dedicated microarray-based platform that uses the Affymetrix Meg Allele technology. The Pain Panel assesses 3,295 single-nucleotide polymorphisms (SNPs) representing 358 genes known to be involved in systems relevant to pain perception (complete list provided through http://www.algynomics.com/pain-research-panel.html). Pathways assessed by the Pain Panel represent 1 or more of 3 broad domains and include genes that 1) mediate the transmission of pain signals by sensory nerve fibers and by central nervous system neural pathways that mediate the perception of pain, 2) mediate peripheral and central inflammatory responses to tissue injury or psychological stress, and 3) influence mood and affective states associated with chronic pain conditions. The Panel also includes genes that influence the pharmacokinetics and dynamics of analgesic compounds and includes ancestry-informative markers. Within each gene, SNPs were prioritized for inclusion on the basis of known functionality (ie, they result in nonsynonymous amino acid changes, expression level differences, or disrupted alternative splicing). Other SNPs were selected as representative markers of regions with high linkage disequilibrium (LD), containing many correlated SNPs that are inherited in blocks, in order to tag untyped SNPs.

Selected duplicate study samples and HapMap reference DNA were genotyped concurrently with each batch in order to examine consistency of genotype calls throughout the study, and genotyping was monitored for batch or site effects in call rates that might introduce bias into the association tests. All samples were clustered together at the conclusion of the genotyping process, using the manufacturer's supplied software in accordance with Affymetrix protocols. Genotyping results were returned for 3,221 unique samples, representing enrollees in the prospective cohort study and in the case-control study. The overall call rate was 99.1% and repeated sample concordance was 99.8%.

Raw genotypes were filtered for quality using utilities implemented in PLINK v.1.07 (Broad Institute, Cambridge, MA).[8] An identity-by-state analysis was performed using principal components analysis to cluster individuals according to racial heritage by multidimensional scaling (Supplementary e-Fig 1). The first 2 principal components (eigenvectors) were retained for use as covariates representing racial background in the association testing. Samples were dropped from the study because of 1) call rate <.95 (n = 38); 2) duplicate genotypes (n = 20); 3) cryptic relatedness (n = 84); 4) mismatch between genotypic and self-reported sex and race (n = 29); and 5) case misclassification (n = 14). SNPs were filtered for 1) call rate <.95 (n = 170); 2) repeated sample concordance rate <.99 (n = 58); 3) minor allele frequency in the full cohort <1% (n = 101); and 4) Hardy-Weinberg equilibrium P value $<1 \times 10^{-5}$ in either non-Hispanic whites or African Americans separately (n = 42). The final cleaned data set included 2,924 SNPs assayed in 3,050 subjects, with a completeness rate of 99.7%. The analytic data set, after removing subjects enrolled as cases at baseline, included 2,536 subjects.

Follow-Up and Case Classification of First-Onset TMD

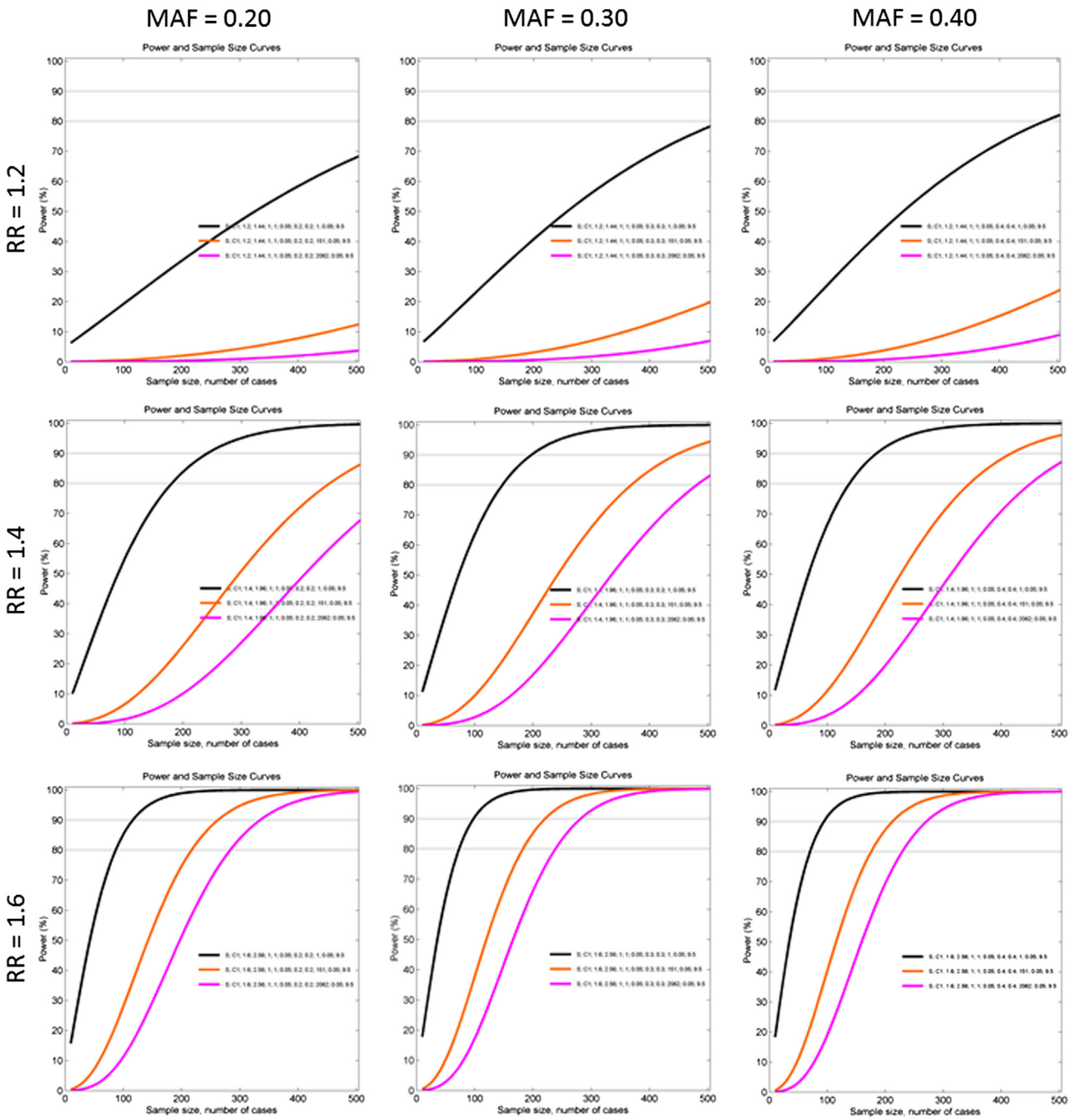
From the time of enrollment through May 2011, study participants were asked to complete a quarterly questionnaire that screened for TMD pain symptoms experienced during the preceding 3 months. Those who reported TMD pain symptoms were asked to attend a clinical examination that determined presence or absence of painful TMD according to Research Diagnostic Criteria for TMD.[3] Classification of first-onset TMD required 2 criteria: 1) ≥5 days/month of pain in TMD locations specified by examiner and 2) examiner findings of arthralgia, myalgia, or both. Arthralgia was

based on pain in temporomandibular joint(s) during jaw maneuver or digital palpation, and myalgia was based on pain during jaw maneuver or digital palpation in ≥3 of 8 muscle groups, each assessed bilaterally: temporalis, masseter, lateral pterygoid, and submandibular.

All examiners underwent annual training and calibration in Research Diagnostic Criteria for TMD. In blinded, replicated examinations, Kappa statistics for interexaminer reliability of TMD case classification ranged from 0.84 to 1.0, signifying excellent reliability.

Statistical Analysis

The follow-up period for each study participant was computed as the time from enrollment to the first of 3 possible events: 1) examiner classification of first-onset TMD; 2) loss to follow-up; or 3) the census date used for this analysis (ie, May 2011). The rate of first-onset TMD was calculated as the number of people with first-onset TMD divided by the sum of follow-up periods, and the result was expressed as the percentage of people per annum (equivalent to the number of incident cases per 100



Supplementary e-Figure1. Power curves for 3 association test conditions: 1) a single unadjusted association test (*black line*); 2) the analysis of a priori selected "first tier" genes (n = 211 SNPs, *orange line*); and 3) the analysis of the full set of genes (n = 2,924 SNPs, *purple line*). The latter 2 analyses are corrected for the number of effectively independent SNPs, after accounting for LD between nearby loci by the spectral decomposition method[4,6] in the tier 1 (n = 151 SNPs) and full set (n = 2,062 SNPs). Figures show the results of power calculations (performed using the PGA power calculator for genetic association studies[5]) assuming relative risks of 1.2, 1.4, and 1.6 and MAFs of .2, .3, and .4, as noted for each row and column, respectively.

years of follow-up). For descriptive purposes, an adjusted annual incidence rate was computed using a Poisson regression model that adjusted for study site using the Buffalo study site as the referent from among the 4 study sites.

To test hypotheses about associations between genetic risk factors and the TMD incidence rate, hazard ratios were computed using Cox proportional hazard regression. In Cox models, incident cases were regarded as an event; otherwise, people were censored. Each person's follow-up period was used as the time-to-event. When calculating univariate hazard ratios and 95% confidence interval models, we adjusted only for study site and genotypic race, using the first 2 eigenvectors as covariates as described above to account for racial stratification.[7]

Because of the large number of potential genetic risk factors tested, it was necessary to correct for the large number of multiple comparisons. The customary Bonferroni correction is known to be overly conservative, because of LD structure between neighboring SNPs. We first calculated the number of effectively independent SNPs in each tier, taking LD structure into account, by the spectral decomposition method,[4,6] before applying the Bonferroni correction. The number of independent SNPs in the tier 1 analysis was estimated to be 151 (from the 211 that passed quality control filters), for a corrected threshold for significance of $P < 3.4 \times 10^{-4}$. For the larger tier 2 SNP set, the number of effectively independent SNPs was 1,911 (of 2,657 total), giving a corrected threshold of $P < 2.6 \times 10^{-5}$.

Sample Size Considerations

OPPERA was designed with a target sample size of 3,200 enrolled study participants expected to yield 196 cases of first-onset TMD during a 3-year follow-up period, assuming 30% loss to follow-up. These targets were based on incidence and cohort retention rates observed in a previous study conducted at the North Carolina study site[2] and were sufficient to provide a statistical power of 80% to risk ratios of at least 1.8 for risk predictors with as few as 15% of people in the high-risk category, consistent with the magnitude of effect seen for genetic predictors seen in the previous North Carolina study. The actual analysis performed included 260 onset cases of TMD and 2,477 subjects that did not develop TMD during the follow-up period. Power curves showing the expected power for this cohort at different levels of relative risk and MAF are shown in Supplementary e-Fig 1.

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