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Genome Wide Association Study of Neuropathic Ocular Pain

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Purpose: To conduct a genome-wide association study (GWAS) of individuals with neuropathic ocular pain (NOP) symptoms to identify genomic variants that may predispose to NOP development.

Design: Prospective study of individuals with NOP.

Participants: Three hundred twenty-nine patients recruited from the Miami Veterans Affairs eye clinic.

Methods: The Neuropathic Pain Symptom Inventory modified for the eye (NPSI-Eye) was completed to calculate a NPSI-Eye-Sub-Score (summed ratings of burning and wind sensitivity) as an indicator of NOP severity. A GWAS was performed for the NPSI-Eye-Sub-Score with a significance threshold of $P < 5 \times 10^{-8}$. A gene-based analysis was performed using the multimarker analysis of genomic annotation software (in the functional mapping and annotation of GWAS online platform). The 13 865 778 single nucleotide polymorphisms (SNPs) from our GWAS analysis were mapped to 10 834 protein coding genes, and significant genes were run through gene set enrichment analysis.

Main Outcome Measures: Identification of SNPs and protein products that may be associated with the development of NOP.

Results: One hundred seventy-one SNPs reached a threshold of $P < 10^{-5}$, of which 10 SNPs reached the suggestive level of significance of $P < 5 \times 10^{-7}$ and 1 SNP met our genome-wide significance threshold of $P < 5 \times 10^{-8}$. This lead SNP, rs140293404 ($P = 1.23 \times 10^{-8}$), is an intronic variant found within gene ENSG00000287251 coding for transcript ENST00000662732.1. Rs140293404 is in linkage disequilibrium with exon variant rs7926353 ($r2 > 0.8$) within ENSG00000279046 coding for transcript ENST00000624288.1. The most significant genes from gene-based tests were matrix metalloproteinase-19 (*MMP19*) ($P = 1.12 \times 10^{-5}$), zinc finger RNA-binding motif and serine/arginine rich-1 (*ZRSR1*) ($P = 1.48 \times 10^{-4}$), *CTC-487M23.8* ($P = 1.79 \times 10^{-4}$), receptor expression-enhancing protein-5 (*REEP5*) ($P = 2.36 \times 10^{-4}$), and signal recognition particle-19 (*SRP19*) ($P = 2.56 \times 10^{-4}$). From gene set enrichment analysis, the sensory perception (false discovery rate $= 6.57 \times 10^{-3}$) and olfactory signaling (false discovery rate $= 1.63 \times 10^{-2}$) pathways were enriched with the most significant genes.

Conclusions: Our GWAS revealed genes with protein products that may impact sensory perception, lending biological plausibility to a role for SNPs identified by our GWAS in the development of NOP. A better understanding of the biological relevance of these genes and pathways in the pathophysiology associated with NOP may facilitate future novel mechanism-based treatments.

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Ocular surface complaints are prevalent in the general population, with an estimated frequency of 5%–30% worldwide.[1] These complaints include pain symptoms, often described as "dryness," "aching," and "tenderness," to name a few, and visual symptoms, such as poor or fluctuating vision.[2] Ocular surface pain complaints have generally been incorporated under the umbrella term "dry eye (DE)," which is defined as "a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."[3] While tear abnormalities have long been recognized as contributors to ocular surface pain, nerve dysfunction (i.e., neuropathic pain) is now recognized as another important contributor.[4,5]

The diagnosis of a neuropathic contribution to ocular surface pain is made clinically. Similar to neuropathic pain

outside the eye,[6] individuals often characterize their pain as "burning" and endorse evoked pain (i.e., to wind and light).[2,7] In addition, individuals with a suspected neuropathic component to pain often have a disconnect between pain complaints and objective ocular surface signs, with symptoms that outweigh signs[8,9] and fail to adequately respond to treatments aimed at improving tear health.[10] Furthermore, these individuals often have comorbid pain conditions, such as migraine,[11] fibromyalgia,[12] and pelvic pain.[13] In fact, we have shown that individuals suffering from multiple chronic pain conditions reported more severe ocular surface pain, but have similar tear parameters, to individuals without multiple pain conditions.[14] Accompanying symptoms in this patient population include mood disorders, disrupted sleep, decreased energy, difficulty concentrating, and an overall decrease in enjoyment of life.[5]

Evidence suggests that genetic contributors may underlie the noted associations between chronic pain conditions and neuropathic ocular surface pain (NOP). Crosstwin crosstrait correlations of DE were found to be higher in monozygotic twins compared to dizygotic twins, suggesting an underlying genetic contribution.[15] Specifically, DE symptoms (which incorporate ocular surface pain) showed a heritability of 29% (95% confidence interval, 18%−40%).[15] Similarly, twin studies of individuals with chronic pain conditions outside the eye, including temporomandibular disorder, tension headache, migraine headache, chronic back pain, and chronic joint pain, reported similar findings, with higher heritability in monozygotic compared to dizygotic twins.[16] Genome wide association studies (GWAS) have been performed to gain additional insight into potential molecular mechanisms that underlie individual susceptibility to migraine,[17] fibromyalgia,[18] and chronic pain.[19] These results have provided information into various nervous system pathways that may be involved in pain persistence. Given the co-existence of NOP with various chronic pain conditions, it is reasonable to assume there are genomic variants that may predispose an individual for NOP development as well. Therefore, we undertook a GWAS on individuals with NOP symptoms in order to identify genomic patterns that in the future may be leveraged to improve patient stratification and be amenable to mechanism-based therapeutic interventions. Given the frequency of ocular pain symptomatology in the population and its negative impact on quality of life, a better understanding of contributors to ocular surface pain are needed, in order to provide precision-based treatment algorithms to an individual patient.

Methods

Study Population

Patients with normal eyelid, conjunctival, and corneal anatomy were prospectively recruited from the Miami Veterans Affairs Medical Center eye clinic between October 2013 and October 2017 and underwent a complete ocular surface examination. Patients were excluded from participation if they wore contact lenses, underwent refractive surgery, used ocular medications with the exception of artificial tears, had human immunodeficiency virus, sarcoidosis, graft versus host disease or a collagen vascular disease (including Sjögrens), had an active external ocular process, or had cataract surgery within the last 6 months or any glaucoma or retinal surgery. Miami Veterans Affairs institutional review board approval allowed the prospective evaluation of patients and informed consent was obtained. The study was conducted in accordance to the principles of the Declaration of Helsinki.

Data Collected

For each individual, demographic information, past ocular and medical history, and medication information were collected.

Comorbidities

Mental health indicators were assessed including the Patient Health Questionnaire 9 regarding depression symptoms (score 0−27)[20] and the Post Traumatic Stress Disorder Checklist − Military Version regarding Post Traumatic Stress Disorder symptoms (score 0−68).[21,22]

Ocular Pain Symptoms

Subjects filled out several standardized questionnaires regarding DE symptoms, including the 5 Item DE Questionnaire[23] (range 0−22) and the Ocular Surface Disease Index (OSDI)[24] (range 0−100). Subjects also filled out ocular pain specific questionnaires, including a numerical rating scale ("How would you describe the overall intensity of your ocular pain on average during the past 1 week?") (range 0−10) and the Neuropathic Pain Symptom Inventory modified for the eye (NPSI-Eye)[25] (range 0−100) (Table S1). This questionnaire was chosen as it has been validated and used in a number of patient populations with various neuropathic pain conditions.[6,26−29] The NPSI consists of 10 scored items that help identify and assess the severity of spontaneous and paroxysmal pain, paresthesias, allodynia, and hyperalgesia. In order to modify the NPSI so that it was relevant to NOP, we replaced 3 of the original questions regarding the severity of allodynia or hyperalgesia caused by (1) light touch, (2) pressure, or (3) contact with something cold on the skin, with questions specific to *ocular* allodynia or hyperalgesia (eye pain caused or increased by [1] wind, [2] light, and [3] heat or cold). A total NPSI-Eye score was calculated, along with an NPSI-Eye-Sub-Score (summed ratings of burning and wind sensitivity), as an indication of the severity of neuropathic-like ocular pain.

Ocular Surface Examination

All individuals underwent tear film assessment including (1) tear breakup time (TBUT) (5 μl fluorescein placed, 3 measurements taken in each eye and averaged), (2) corneal staining (National Eye Institute scale, 5 areas of cornea assessed [score 0−3 in each]),[30] (3) Schirmer's strips with anesthesia and eyelid and Meibomian gland assessment.[31] Subjects also underwent testing of corneal sensitivity with a modified Belmonte esthesiometer.[32] For corneal detection threshold measurements, subjects were presented with a stimulus immediately following a blink, and asked to indicate whether they felt the stimulus by pressing a button. The initial flow rate was set at a level below threshold (30 mL/minute for most individuals) and increased by 10 mL/minute (with 15-second intervals between stimuli) until the subject stated that they felt the stimulus, or the maximum allowable flow rate (400 mL/minute) was reached. Two ascending series were conducted and detection threshold was defined as the arithmetic mean of the value at which the subject pressed the button across the 2 series. To estimate ocular pain threshold, the flow rate was further increased

beyond the detection threshold in 10 mL/minute increments until the subject reported the stimulus as painful or the maximum allowable flow rate (400 mL/minute) was reached. Two ascending series were conducted in this way and pain threshold was defined as a mean of the 2 series.

Genotyping and Genetic Association Analysis

Genomic DNA was purified from whole blood using Puregene chemistry on the Qiagen Autopure LS according to standard automated Qiagen protocols. Samples were genotyped using Illumina's Infinium Expanded Multiethnic Genotyping Array (MEGA^{EX}) that interrogates approximately 2 million markers. The samples were processed according to Illumina Procedures for processing of the Infinium LCG Assay. Data were extracted by the Illumina Genome Studio software from data files created by the Illumina iScan. Samples with call rates $< 98\%$ were excluded from analysis and a GenCall cutoff score of 0.15 is used for all Infinium II products. Imputation was performed using the Michigan Imputation Server with the 1000 Genomes reference population. After initial quality control, which consisted of the removal of monomorphic variants and single nucleotide polymorphisms (SNPs) with $> 10\%$ missing rate, 1 304 222 autosomal SNPs remained. Further filtering was conducted during preimputation evaluation to compare SNPs with 1000 Genomes reference, resulting in 1 162 578 SNPs available for submission to the Imputation Server. The data returned from the Michigan Imputation Server was filtered to exclude monomorphic SNPs and those with INFO/r2 values < 0.4, resulting in 43 392 883 SNPs available for analysis. We then performed a GWAS in 329 individuals using an NPSI-Eye-Sub-Score (summed ratings of burning and wind sensitivity) as the outcome, adjusting for age, sex, and the first 3 principal components. The frequentist model using a score test in the program SNPTEST version 2.5.6 was used to test for association and models were run with and without Hardy-Weinberg Equilibrium correction. Single nucleotide polymorphisms were then filtered for minor allele frequency (MAF) > 0.01 and imputation info score > 0.8, resulting in a final number of 13 865 778 SNPs. A genome wide significance threshold of $P < 5 \times 10^{-8}$ was used. Annotation was performed for the nearest genes $\pm$ 1 million base pairs (bps) from each SNP.

Gene- and Set-Based Analyses

A gene-based analysis was performed using the multimarker analysis of genomic annotation software (in the functional mapping and annotation online platform). Input SNPs from our GWAS analysis were mapped to 10 834 protein coding genes, using a gene-based significance threshold of 4.61×10^{-6}. The top 25 genes were also run through gene set enrichment analysis[33,34] to discover additional relevant pathways.

Statistical Analysis of Phenotype

Descriptive statistics were computed to summarize patient demographic and clinical information. The distributions of spread of DE symptoms and signs were assessed. Participants were grouped by the presence of heterozygosity for rs140293404 and/or SNPs in linkage disequilibrium (LD) and differences in DE metrics between the groups were assessed. To examine symptom/sign discordance, a delta (Δ) value was determined using 3 symptom parameters (Dry Eye Questionnaire 5, OSDI, and NPSI-Eye total) and 3 sign parameters (TBUT, corneal staining, and Schirmer's). Each parameter was normalized to a value between 0 (no symptoms/signs) and 1 (most severe symptoms/signs). A total symptom/sign score was calculated by averaging the normalized values of the 3 symptom/sign parameters. The Δ was determined by calculating the difference between the normalized total symptom score and the normalized total sign score. The Δ ranged from -1 (minimal symptoms and maximal signs) to 1 (maximal symptoms and minimal signs). A more positive Δ implies more discordance between reported symptoms and ocular signs observed on exam, where symptoms outweigh signs (symptoms $>$ signs). Mann-Whitney tests were used to examine differences between the groups for continuous variables. Spearman rho values were calculated for correlational analyses. Statistical analysis was performed using SPSS, version 28.0 (IBM Corp).

Results

Study Population

Three hundred twenty-nine patients participated in the study. The mean age of participants was 61.8 years old with a standard deviation of 9.8 years; the majority of individuals were male (92%), Black (56%), and non-Hispanic (79%). Sixty-five percent of individuals had a Patient Health Questionnaire 9 score indicative of mild or greater depressive symptoms (≥ 5), with a mean score of 9.4 ± 7.8. The mean NSPI-Eye total score was 20.2 ± 21.4. The NPSI-Eye-Sub-Score mean for summed ratings of burning and wind sensitivity was 5.1 ± 5.4. Both measures of NOP were significantly correlated $P < 0.001$, rho $= 0.89$ (Fig S1). Dry eye symptoms and signs varied among the participants (Fig S2). Demographics, co-morbidities, ocular symptoms, and DE signs of the study population are displayed in Table 2.

Genetic Loci Associated with NOP

To identify genetic loci associated with NOP as assessed by NPSI-Eye-Sub-Score (burning and wind sensitivity), we performed a GWAS analysis. One hundred seventy-one SNPs reached a threshold of $P < 10^{-5}$ (Table S3), of which 10 SNPs reached a suggestive level of significance of $P < 5 \times 10^{-7}$ (Table 4) and 1 lead SNP met our genome wide significance threshold of $P < 5 \times 10^{-8}$ (Fig 3). The SNP rs140293404 is an intronic variant (ancestral allele C) on chromosome (chr) 11 at 134 968 716 bp (forward strand, Genome Reference Consortium Human Build 38, within the transcript of a newly discovered long intergenic noncoding RNA (lincRNA) (ENST00000662732.1), a product of gene ENSG00000287251 (chr 11:134,815,012-134,850,468 bp). Interestingly, rs140293404 has a MAF of 0.02 and 0 in European and African populations, respectively, but was found to have a MAF of 0.03 in our study population.

Predicted Consequences of Lead SNPs

Our lead SNP, which was imputed with INFO $= 0.816$, is predicted to alter the binding motif of transcription factor Zbtb3. Expression of this protein includes nervous tissues consistent with a potential pathogenic role in ocular surface pain.[35,36] Exploring this 2322 bp transcript (ENST00000662732.1) further shows $\geq 95\%$ identity in alignment with chr 18: 718 bp (linc01443; 18:14,973,452-14,974,238), 280 bp (18: 14,942,385-14,942,691), 27 bp (18: 14,942,370-14,942,399); chr 3:

Table 2. Demographics, Comorbidities, and Ocular Symptoms and Signs in the Study Population

Demographics	
Age, years, mean ± SD (range)	61.78 ± 9.79 (32–91)
Gender, male, n (%)	302 (92)
Race, White, n (%)	145 (44)
Black, n (%)	183 (56)
Ethnicity, Hispanic, n (%)	70 (21)
Comorbidities	
Hypertension, n (%)	229 (70)
Diabetes mellitus, n (%)	99 (30)
Sleep apnea, n (%)	74 (22)
Benign prostatic hypertrophy, n (%)	56 (17)
Former smoker, n (%)	154 (47)
Current smoker, n (%)	130 (40)
Migraine, n (%)	45 (14)
Depression symptoms, PHQ-9, mean ± SD (range)	9.38 ± 7.83 (0–27)
PTSD symptoms, PCL-M, mean ± SD (range)	39.64 ± 19.34 (17–85)
Ocular symptoms, mean ± SD (range)	
DE symptoms assessed via DEQ5	11.24 ± 5.18 (0–22)
DE symptoms assessed via OSDI	34.91 ± 24.35 (0–100)
Ocular pain assessed via NRS average over past week	3.18 ± 2.63 (0–10)
Ocular pain assessed via NRS worst over past week	4.08 ± 3.18 (0–10)
Neuropathic ocular pain assessed via NPSI-Eye, total score	20.17 ± 21.41 (0–100)
NPSI-Eye-Sub-Score (burning + wind sensitivity)	5.11 ± 5.42 (0–20)
DE signs*, mean ± SD (range)	
Tear break up time, seconds	8.95 ± 4.45 (0–28)
Corneal staining	2.05 ± 2.48 (0–14)
Schirmer's test, mm wetting at 5 min	12.98 ± 7.35 (0–41)
Corneal sensation†, mean ± SD (range)	
Detection threshold, ml/min	86.23 ± 44.70 (10–410)
Pain threshold, ml/min	229.05 ± 114.08 (10–410)

DE = dry eye; DEQ5 = Dry Eye Questionnaire 5; NPSI-Eye = Neuropathic Pain Symptom Inventory Modified for the Eye; NRS = Numerical Rating Scale; OSDI = Ocular Surface Disease Index; PCL-M = PTSD Checklist – Military Version; PHQ-9 = Patient Health Questionnaire 9; PTSD = posttraumatic stress disorder; SD = standard deviation.
*More abnormal value between the 2 eyes for each patient was included in the analysis.
†Corneal sensation taken from the right eye.

(3:173,525,747-173,525,772 reverse strand) that overlaps with the forward strand of protein coding transcripts neuroligin-1 (NLGN1)-204 (ENST00000423427.1), NLGN1-211 (ENST00000695368.1), NLGN1-202 (ENST00000413821.1), and NLGN1-205 (ENST00000457714). In addition, bp 1995–2019 of ENST00000662732.1 aligns with the reverse strand of growth hormone receptor gene (ENSG00000112964), overlapping with all known transcripts coded on the forward strand of chr 5: (42,673,744-42,673,770).

This lead SNP is in complete LD (r = 1.0, D' = 1.0) with SNP rs7926353 chr 11:134,986,732 within gene ENSG00000279046 (forward strand, Genome Reference Consortium Human Build 38 134,985,683-134,986,799), which is an exon variant (position 1050 out of 1117) within transcript ENST0000062488.1 (MAF: 0.02, ancestral allele

Table 4. List of the Top SNPs Found to Be Associated With Neuropathic Ocular Pain Symptoms via Genome Wide Association Study

Chr	Position (GRCH38)	rsid	Cohort MAF	*P* Value	Functional Consequence	Genes
11	134968716	rs140293404	0.03	1.23E-08	intronic	Lnc-B3GAT1-2 (ENSG00000287251)
12	115412447	rs61931467	0.03	8.41E-08	intergenic	TBX3, MED13L
18	26229978	rs74942254	0.17	1.36E-07	intronic	TAF4B
2	41795441	rs78163327	0.045	2.30E-07	intergenic	SLC8A1, LOC388942
7	106585971	rs60979431	0.02	2.85E-07	intergenic	NAMPT, CCDC71L
1	208763936	rs6701123	0.16	4.00E-07	intergenic	PLXNA2, MIR205HG
19	1720333	rs4807953	0.33	4.10E-07	intergenic	TCF3, ONECUT3
1	109855735	rs144685507	0.02	4.60E-07	intergenic	EPS8L3, CSF1
6	147726229	rs113491581	0.02	4.73E-07	intergenic	SAMD5, SASH1
1	208764239	rs11585407	0.004	4.98E-07	intergenic	PLXNA2, MIR205HG

Chr = chromosome; MAF = minor allele frequency; rsid = reference SNP cluster ID; SNP = single nucleotide polymorphism.

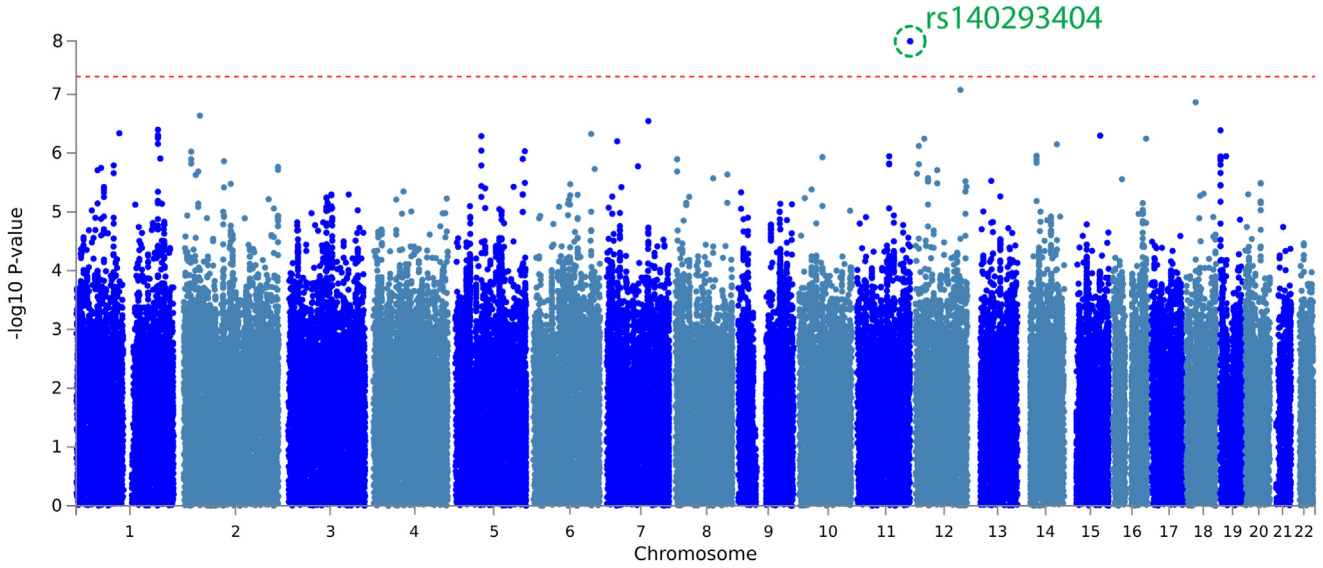

Figure 3. Manhattan plot of single nucleotide polymorphisms (SNPs) associated with neuropathic ocular pain assessed by Neuropathic Pain Symptom Inventory modified for the eye (NPSI-eye)-sub-score. X-axis, chromosome position; Y-axis, −log10 (*P* value) after linear regression for NPSI-eye-sub-score and SNP dosage, correcting for age, sex, and the first 3 principal components of genetic variation. Each dot represents a SNP tested in the association test. Horizontal dashed line represents the significance threshold, $P < 5 \times 10^{-8}$.

A). rs140293404 is also in LD with rs114671494 and rs116112008 ($r^2 > 0.8$), of which rs116112008 is predicted to alter epigenetic patterns impacting enhancer formation in stem cells and brain tissue.

These genes and transcripts lie entirely within a region of the genome densely populated by copy number variants. Some copy number variants are associated with deletion or duplication of multiple genes that may contribute to the NOP phenotype. For example, nsv4194329 was associated with the deletion (nsv4206273; Table S5) and the duplication of multiple transcripts (nsv4194329; Table S6; and nsv4528794; Table S7).

Loci Associated with DE Metrics

Dry eye metrics were assessed in all patients using both symptoms and signs. Overall, individuals who were heterozygous for the lead SNP, rs140293404 (56.2 ± 23.6 vs. 34.4 ± 24.1, $P = 0.01$), and/or SNPs in LD (rs114671494, 116112008, and rs7926353) (lead SNP+LD) (47.3 ± 26.5 vs. 34.3 ± 24.0, $P = 0.03$) had significantly greater DE symptoms as measured by the OSDI. Individuals heterozygous for the lead SNP (5.7 ± 1.4 vs. 3.1 ± 2.6, $P = 0.004$) and the lead SNP+LD (4.8 ± 2.2 vs. 3.1 ± 2.6, $P = 0.003$) also had significantly greater ocular pain, reported by the numerical rating scale averaged over the last week. Neuropathic like eye symptoms, as measured by the NPSI-Eye, were also more severe in individuals heterozygous for the lead SNP (39.0 ± 21.6 vs. 19.6 ± 21.2, $P = 0.009$) and the lead SNP+LD (36.2 ± 26.9 vs. 19.2 ± 20.7, $P = 0.007$). While DE signs were similar across the groups, individuals heterozygous for the lead SNP (0.05 ± 0.2 vs. −0.2 ± 0.2, $P = 0.01$) or lead SNP+LD (0.07 ± 0.2 vs. −0.2 ± 0.2, $P < 0.001$) had a significantly greater Δ between symptoms and signs, where symptoms outweighed signs (symptoms > signs) (Table 8).

Gene-based and Pathway Analyses

To further investigate these enriched regions, we performed a gene-based test of association utilizing multi-marker analysis of genomic annotation through functional mapping and annotation. Input SNPs were mapped to 10 834 protein coding genes. The top genes associated with our GWAS were matrix metalloproteinase-19 (*MMP19*) ($P = 1.12 \times 10^{-5}$), zinc finger RNA-binding motif and serine/arginine rich-1 (*ZRSR1*) ($P = 1.48 \times 10^{-4}$), *CTC-487M23.8* ($P = 1.79 \times 10^{-4}$), receptor expression-enhancing protein-5 (*REEP5*) ($P = 2.36 \times 10^{-4}$), and signal recognition particle-19 (*SRP19*) ($P = 2.56 \times 10^{-4}$) (Fig 4) (Table 9); no gene reached the significance threshold of 4.61×10^{-6}.

Gene set enrichment analysis of reactome pathways was performed with the top 25 genes. Strikingly, sensory perception (false discovery rate $= 6.57 \times 10^{-3}$) and olfactory signaling (false discovery rate $= 1.63 \times 10^{-2}$) pathways were both enriched with top genes (Table 10).

Discussion

This study evaluated associations between genetic loci and symptoms of NOP. In our pilot cohort of 329 individuals, we found that 1 SNP, rs140293404, reached genome wide significance ($P < 5 \times 10^{-8}$) with a MAF of 0.03. Rs140293404 is an intronic variant found within gene ENSG00000287251 that codes for a 2322 bp lincRNA (ENST00000662732.1). BLAST-like alignment tool of this transcript aligns with a lincRNA (LINC01443), and the noncoding strands of *NLGN1* and growth hormone receptor.

Table 8. Dry Eye Symptoms and Signs in Individual Groups by Heterozygosity for the Lead SNP and/or SNPs in LD

DE Metric [Range]	Lead SNP (n = 9) (Mean ± SD)	Lead SNP Control (n = 320) (Mean ± SD)	Mann Whitney U	Lead SNP *P* Value	Lead SNP + LD (n = 19) (Mean ± SD)	Lead SNP + LD Control (n = 310) (Mean ± SD)	Mann Whitney U	Lead SNP + LD *P* Value
Symptoms								
DEQ5, [0–22]	14.6 ± 3.0	11.1 ± 5.2	891.5	0.05	14.3 ± 4.0	11.0 ± 5.2	1818.0	0.005*
OSDI, [0–100]	56.2 ± 23.6	34.4 ± 24.1	711.5	0.01*	47.3 ± 26.5	34.3 ± 24.0	2062.5	0.03*
NRS right now, [0–10]	4.8 ± 3.0	2.7 ± 2.6	828.0	0.03*	4.2 ± 3.0	2.6 ± 2.6	1994.5	0.02*
NRS average over past week, [0–10]	5.7 ± 1.4	3.1 ± 2.6	635.0	0.004*	4.8 ± 2.2	3.1 ± 2.6	1776.5	0.003*
NRS worst over past week, [0–10]	7.8 ± 1.8	4.0 ± 3.2	469.5	< 0.001*	6.4 ± 3.0	3.9 ± 3.2	1615.0	< 0.001*
NPSI-Eye total, [0–100]	39.0 ± 21.6	19.6 ± 21.2	700.0	0.009*	36.2 ± 26.9	19.2 ± 20.7	1850.0	0.007*
Signs†								
TBUT, sec	9.5 ± 4.7	8.9 ± 4.5	1308.0	0.66	11.0 ± 5.7	8.8 ± 4.3	2255.0	0.09
Corneal staining, [0–14]	1.4 ± 2.1	2.1 ± 2.5	1201.0	0.39	1.4 ± 2.1	2.1 ± 2.5	2449.5	0.21
Schirmer's test, mm wetting at 5 min	12.3 ± 7.3	13.0 ± 7.4	1346.0	0.75	16.8 ± 10.0	12.7 ± 7.1	2234.5	0.08
Δ Between Symptoms and Signs								
Δ Value	0.05 ± 0.2	−0.2 ± 0.2	736.0	0.01*	0.07 ± 0.2	−0.2 ± 0.2	1402.0	< 0.001*
Corneal sensation via Belmonte Aesthesiometer (taken from the right eye)								
Detection threshold ml/min [10–410]	68.3 ± 28.6	86.7 ± 45.1	1064.0	0.21	73.2 ± 32.0	87.0 ± 45.4	2351.0	0.17
Pain threshold ml/min [10–410]	197.2 ± 127.2	230.0 ± 114.0	1104.0	0.35	222.4 ± 119.2	229.5 ± 114.1	2623.0	0.73

DE = dry eye; DEQ5 = Dry Eye Questionnaire 5; LD = linkage disequilibrium; NPSI-Eye = Neuropathic Pain Symptom Inventory modified for the eye; NRS = Numerical Rating Scale; OSDI = Ocular Surface Disease Index; SD = standard deviation; SNP = single nucleotide polymorphism; TBUT = tear break up time.
Δ Calculated as the difference between normalized symptoms (average of DEQ5, ODSI, and NPSI-Eye total) and signs (average of TBUT, corneal staining, and Schirmer's).
*Statistically significant difference at *P* value < 0.05.
†More abnormal value between the 2 eyes for each patient was included in the analysis.

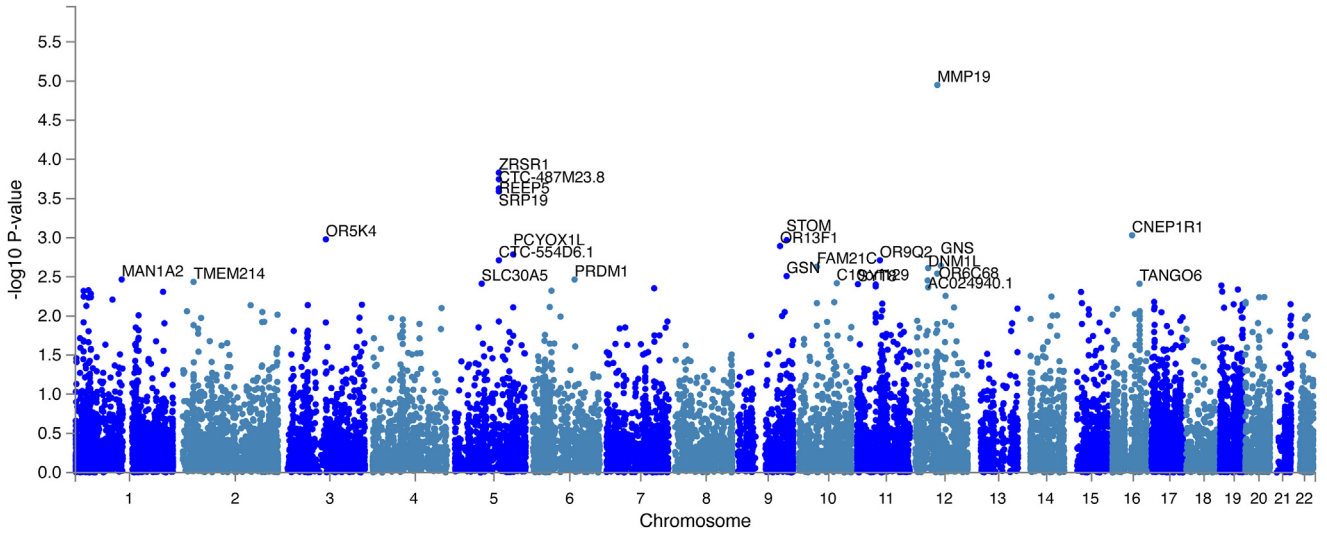

Figure 4. Manhattan plot of the gene-based test as computed by multimarker analysis of genomic annotation analysis using genome wide association study summary statistics. X-axis, chromosome position; Y-axis, −log10 (*P* value). Each dot represents a gene tested. MMP19 = matrix metalloproteinase-19.

We also found that rs140293404 is in complete LD with rs7926353, an exon variant within the adjacent transcript ENST00000624288.1. BLAST-like alignment tool of this transcript aligns with the noncoding strands of zinc finger and SCAN domain containing 5A and protein phosphatase-2 catalytic subunit beta. Our lead SNP is also in LD with 2 other SNPs: rs114671494 and rs116112008, where rs116112008 is predicted to impact epigenetic mechanisms that alter enhancer formation in specific tissues, including stem cells and brain tissue. These SNPs are located in a region densely populated by copy number variant. These genomic regions have been described as able to impact gene regulation through a variety of mechanisms, including altering transcript binding and repression of gene expression.[37] The collective analysis of our gene-based association test revealed specific protein coding genes that may be influenced by the SNPs in our GWAS. In particular, the top genes associated were *MMP19*, *ZRSR1*, *CTC-487M23.8*, *REEP5*, and *SRP19*. The protein products of these genes could impact sensory perception and olfactory signaling, lending biological plausibility to a potential impact of rs140293404 on NOP development.

The idea that genetic polymorphisms may underlie aspects of DE (symptoms and signs) is supported by prior work. One study obtained blood samples from Korean patients with non-Sjogren's aqueous tear deficiency dry eye disease (DED) (defined as OSDI score $\geq$ 25, Schirmer's test $<$ 5 mm/5minute, TBUT $<$ 5 seconds, and corneal punctate fluorescein staining $\geq$ 1; n = 251) and controls (n = 109) to investigate variations in the proinflammatory cytokine genes interleukin-1B (*IL1B*), interleukin-6 (*IL6*), and and interleukin-6R (*IL6R*).[38] rs1143634 within *IL1B* was significantly different between cases and controls, with an increased frequency of the C/T genotype in cases (10.4% vs 3.9%, $P = 0.04$, odds ratio [OR] = 3.34). A difference in rs8192284 within *IL6R* was also noted between the groups in the C/C genotype (24.3% vs. 15.9%, $P = 0.02$, OR = 2.12) and C allele (OR = 1.26).[38] Another study investigated variants in *MUC1*, a transmembrane mucin with anti-inflammatory properties, in White females with aqueous tear deficiency DED (defined as Schirmer's test $<$ 7 mm/5minute, TBUT $<$ 10 seconds, and ocular surface staining; n = 32) and evaporative DED (defined as TBUT $<$ 7 seconds, Schirmer's test $>$ 7 mm/5minute and meibomian gland obstruction; n = 21) compared to controls (n = 29).[39]

Table 9. Top 25 Genes From the Multimarker Analysis of Genomic Annotation Software

Chr	Start	Stop	*P* Value	Symbol
12	55835433	55842966	1.12E-05	MMP19
5	112891610	112893097	1.48E-04	ZRSR1
5	112861188	112893079	1.79E-04	CTC-487M23.8
5	112876385	112922289	2.36E-04	REEP5
5	112861188	112898371	2.56E-04	SRP19
16	50024410	50037088	9.30E-04	CNEP1R1
3	98353854	98354819	1.05E-03	OR5K4
9	121338987	121370304	1.08E-03	STOM
9	104504263	104505222	1.28E-03	OR13F1
5	149358037	149369653	1.63E-03	PCYOX1L
5	112827213	112867582	1.94E-03	CTC-554D6.1
11	58189070	58194053	1.94E-03	OR9Q2
12	64713445	64759431	2.28E-03	GNS
10	45727043	45792964	2.35E-03	FAM21C
12	32679200	32745650	2.44E-03	DNM1L
12	55492363	55493316	2.87E-03	OR6C68
9	121201483	121332843	3.09E-03	GSN
1	117367449	117528872	3.41E-03	MAN1A2
6	105992690	106109939	3.42E-03	PRDM1
12	31324315	31325945	3.52E-03	AC024940.1
2	27032910	27041694	3.67E-03	TMEM214
10	95194200	95228929	3.80E-03	C10orf129
5	69093991	69131069	3.86E-03	SLC30A5
16	68843531	69085182	3.87E-03	TANGO6
11	1828307	1837521	3.93E-03	SYT8

Chr = chromosome; MMP19 = matrix metalloproteinase-19.

Table 10. Top Pathways From Gene Set Enrichment Analysis for Significant Genes After Multimarker Analysis of Genomic Annotation Analysis

Gene Set Name	P-Value	FDR
Reactome sensory perception	3.97E-6	6.57E-3
Reactome olfactory signaling pathway	1.97E-5	1.63E-2

FDR = false discovery rate.

The G/G, A/G, and A/A genotype frequencies of rs4072037 of *MUC1* were all significantly different when comparing controls to the aqueous tear deficiency ($P = 0.02$) and evaporative DED groups ($P = 0.02$). Specifically, the G/G genotype was markedly lower in the aqueous tear deficiency DED group compared to controls (3% vs. 24%). The genotype frequencies between the aqueous tear deficiency and evaporative DED groups were not significantly different ($P = 0.09$).[39] Studies have shown that genetic susceptibility to inflammation influences neuroplasticity and neuronal dysfunction, contributing to neuropathic pain,[40] although the impact of these polymorphisms on ocular surface pain was not specifically tested in these studies. Interestingly, our results showed a significant association between DE symptoms, but not signs, with our genetic marker of NOP, suggesting that different contributors, and perhaps different underlying genetic predispositions, underlie various DE presentations.

A variety of genetic mechanisms may link our noted polymorphism to the development of NOP. First, genomic regions associated with rs140293404 that have potential lincRNA-mRNA binding patterns may impact neuronal gene expression, and thus influence NOP development. Specifically, altered *NLGN1* expression may impact the pathogenesis of neuropathic pain.[41] In an animal model, nerve injury resulted in increased mRNA and protein levels of NLGN1 in excitatory neurons of the spinal dorsal horn. The subsequent knockdown of *NLGN1* expression was then shown to alleviate mechanical allodynia.[41] It is also important to recognize that rs140293404 may influence the epigenetic predisposition of neuronal cells. Our results show that rs140293404 alters the binding motif of transcription factor Zbtb3 at its location and that the SNPs in LD with rs140293404 are predicted to impact enhancer formation in neuronal tissue. Interestingly, in animal models, Zbtb3 was found to play a role in glucocorticoid receptor signaling in a neuronal context, altering eye development and specifically, development of the corneal epithelium, vessels, and stroma.[35,36] Therefore, the impact of the genomic regions associated with rs140293404 on gene expression and epigenetic mechanisms in neuronal tissue may provide insight into the development of NOP.

The second genetic mechanism involves SNPs identified in our GWAS that may influence the expression or function of protein coding genes related to neuropathic pain. In particular, *MMP19* has been previously implicated in multiple sclerosis, a disease that can manifest with neuropathic pain symptoms including extremity pain, trigeminal neuralgia, and Lhermitte's sign.[42] Specifically, 1 study investigating the RNA expression levels of *MMP19* in peripheral venous blood found a trend toward increased levels in multiple sclerosis patients compared to healthy controls (14.68 ± 0.99 vs. 13.84 ± 0.30, $P = 0.05$).[43] Matrix metalloproteinase-19 has also been implicated in the pathogenesis of neuropathic pain. In one study, spinal cord injury was induced in mice and the expression of MMP mRNA in the spinal cord was quantified.[44] Matrix metalloproteinase-19 was found to be upregulated as early as 24 hours after spinal cord trauma and continued to increase 48 hours after injury compared to baseline levels (0.90 and 1.05 fold increase respectively, $P < 0.001$).[44] Therefore, it is possible that allelic variants of rs140293404 may impact *MMP19* susceptibility, promoting neuropathic pain through trigeminal neurons supplying the ocular surface.

Beyond NOP development, our noted genetic polymorphism may shed light on the link between DE and other comorbidities. For example, depression has been closely related to DE symptoms, especially pain.[45,46] In 1 study, individuals were divided based on the presence of NOP features (burning, pain evoked by wind, light, and air temperature; numerical rating scale 0−10) into a low NOP group (n = 130) and a high NOP group (n = 51).[45] Significant differences between the low and high NOP groups were present when comparing depression scores (PHQ9; 7.6 ± 7.2 vs. 13.5 ± 8.1, $P < 0.0005$), but not when comparing ocular surface signs such as TBUT (9.2 ± 3.6 vs. 8.9 ± 4.2 seconds, $P = 0.69$), corneal staining (2.2 ± 2.8 vs. 2.2 ± 2.3, $P = 0.55$) and Schirmer's scores (13.5 ± 6.1 vs. 14.0 ± 7.2 mm of moisture, $P = 0.09$).[45] This suggests that ocular symptoms relate to systemic co-morbidities, including depression, more so than to ocular surface findings.[45–47] Another gene identified in our gene-based association studies, *REEP5*, has been previously studied in patients with major depressive disorder. One study, comparing SNP associations in Chinese major depressive disorder patients who either responded or did not respond to treatment with serotonin reuptake inhibitors, found differences in the *REEP5* gene.[48] Specifically, when comparing responders to nonresponders, differences in the MAF of the A/G genotype of rs496794 (0.33 vs. 0.54, $P = 0.017$, OR = 2.40), the G/A genotype of rs154549 (0.32 vs. 0.53, $P = 0.02$, OR = 2.35), and the A/G genotype of rs153560 (0.29 vs. 0.50, $P = 0.01$, OR = 2.47) within the *REEP5* gene were found.[48] Therefore, the influence of genetic variants, including within the *REEP5* gene, may contribute to both NOP and depression development.

Our findings may also highlight a potential relationship between aspects not typically recognized as being related to neuropathic pain. In particular, the genes revealed by our gene-based association test suggest a possible association between NOP and olfaction. Within our top 25 genes, 4 genes were those of various olfactory receptor families (*OR5K4*, *OR13F1*, *OR9Q2*, and *OR6C68*). Additionally, gene set enrichment analysis revealed olfactory signaling as a top pathway after multi-marker analysis of genomic annotation analysis. Interestingly, olfaction has been

previously investigated in patients with neuropathic pain. In 1 study, a patient with neuropathic, electric shock-like pain of the right arm, and 3 controls without neuropathic pain, were presented with an unpleasant odor and a pleasant odor and asked to rate the level of pain/tingling intensity while undergoing a functional magnetic resonance imaging scan.[49] All 3 of the control subjects rated pain/tingling intensity (scale from 0 to 10, 10 being most intense) as near zero for the unpleasant odor, pleasant odor, and no odor (average: 0.3 vs. 0.3 vs. 0.3), whereas the neuropathic pain patient rated pain/tingling intensity as higher for the unpleasant odor compared to the pleasant odor and no odor (8.7 vs. 5.0 vs. 4.6). On functional magnetic resonance imaging, the neuropathic pain patient had greater activation of regions associated with pain processing including the thalamus, amygdala, insular cortices, and anterior cingulate cortices after the unpleasant odor compared to the pleasant odor. In contrast, none of the controls had significant activations in any of the pain regions after either odor.[49] Neuropathic pain evoked by odor or taste in the trigeminal region has also been previously described. In a case series of 6 patients with recurrent facial pain after surgery, an episodic, electric shock-like pain in the preauricular region was elicited by the smell of food and by the placement of food in the mouth.[50] It was hypothesized that these findings were the result of pathological interactions between parasympathetic salivary efferent fibers and trigeminal sensory afferents, triggering pain in the auriculotemporal sensory nerve region.[50] Activation of the sympathetic nervous system may also underlie the association between olfaction and neuropathic pain. Previous studies have shown that stress can augment neuropathic pain symptoms.[51–53] Additionally, unpleasant odors have been shown to induce stress and activate the sympathetic nervous system, evidenced by an increased startle reflex and heart rate.[54] As such, the olfactory genes and pathways revealed in our NOP population may provide a genetic basis into the possible association between olfaction and neuropathic eye pain.

The genomic regions and protein coding genes revealed in our study have potential treatment implications. The current treatment of NOP includes first addressing nociceptive sources of pain, such as with tear supplements and anti-inflammatory agents. If pain persists, neuromodulators can be considered in the form of topical, oral, or adjuvant therapy. Gabapentin, pregabalin, nortriptyline, and topiramate have all been used in the treatment of NOP, with varying success.[55–57] Adjuvant agents are often added, as appropriate, including botulinum toxin injections[58,59] and noninvasive neurostimulation devices.[60] However, despite these treatment options, pain persists in a number of patients.[61] These results highlight the need for more specific therapy targeting NOP, as the current treatments that may be efficacious in aqueous tear deficiency or evaporative DED are often insufficient in cases of NOP. Identifying genetic polymorphisms related to NOP can lead to the development of new therapeutic approaches. For example, MMP inhibitors have been suggested as a potential therapeutic target for the treatment of neuropathic pain. In a mouse model, MMP-9 mRNA expression was analyzed after sciatic nerve crush.[62] At 1 day after crush, MMP-9 expression was significantly elevated (86.9 ± 7.8 fold, $P < 0.01$) compared to baseline. Additionally, spontaneous pain behavior determined based on the positioning of the injured paw was investigated in MMP-9 knockout mice compared to controls after sciatic nerve crush. The MMP-9 knockout mice were found to have a significantly lower pain index compared to controls at 2 days, 8 days, and 10 days after crush (3.1 vs. 3.5, 2.2 vs. 3.2, 1.2 vs. 2.1, $P < 0.05$).[62] Therapeutics that block MMP have also been examined with respect to neuropathic pain. For example, minocycline is an inhibitor of MMP-2 and MMP-9.[63] In a chronic constriction injury mice model, minocycline (30 mg/kg) was found to significantly decrease tactile allodynia (weight applied to wire before paw withdrawal: 2.0 vs. 0.7 g, $P < 0.001$) and thermal hyperalgesia (time on cold plate: 7.9 vs. 3.9 s, $P < 0.001$) compared to no treatment on day 7 after injury.[64] Minocycline was also shown to potentiate the antiallodynic and antihyperalgesic effect of morphine when administered together.[64] While MMP-2 and MMP-9 have been the main MMPs studied in neuropathic pain, further studies are needed to investigate the role of other MMPs, including MMP19, based on our current findings. Taken together, our study highlights the possibility of translating genetic targets into novel treatments for NOP.

As with all studies, our findings need to be considered in light of our study limitations. First, our sample size was small and consisted of South Florida veterans. Therefore, our findings may not be generalizable to other populations. Second, since our study excluded individuals with graft versus host disease, Sjögren's, and sarcoidosis, we cannot comment on the genomic patterns of NOP in patient populations with comorbid autoimmune diseases. Similarly, individuals who had previous ocular surgeries, beyond cataract surgery, were excluded, and therefore these findings may not be applicable to patients with postsurgical NOP. Lastly, since our population was mostly male, our findings may not be generalizable to cases of NOP with comorbid pain syndromes such as fibromyalgia and pelvic pain, which are more commonly seen in females. Despite these limitations, the impact of our study is the examination of potential genetic contributors that underlie NOP and could be targeted by specific therapies. Future studies with a larger sample size and a more diverse patient population are needed to validate our findings and identify other genetic polymorphisms that may be involved in NOP. Additionally, future work is needed to probe whether rs140293404 or rs7926353 alter either the splicing or stability of ENST00000662732.1 or ENST00000624288.1. It may also be important to explore whether either transcript acts as antisense RNA to these aligned genes, impacting their expression and sensory neuronal functions relevant to NOP symptoms. Considering the negative impact that NOP has on patient function and quality of life, these lines of investigation can lead to the introduction of targeted therapies and improved treatment algorithms.

Footnotes and Disclosures

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No animal subjects were included in this study.

Author Contributions:

Conception and design: Huang, Rodriguez, Slifer, Martin, Levitt, Galor

Analysis and interpretation: Huang, Rodriguez, Slifer, Martin, Levitt, Galor

Data collection: Huang, Rodriguez, Slifer, Martin, Levitt, Galor

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Overall responsibility: Huang, Rodriguez, Slifer, Martin, Levitt, Galor

Abbreviations and Acronyms:
bp = base pair; **chr** = chromosome; **DE** = dry eye; **DED** = dry eye disease; **GWAS** = genome-wide association study; **LD** = linkage disequilibrium; **IL** = interleukin; **lincRNA** = long intergenic noncoding RNA; **MAF** = minor allele frequency; **MMP** = matrix metalloproteinase; **NLGN1** = neuroligin-1; **NOP** = neuropathic ocular pain; **NPSI-Eye** = Neuropathic Pain Symptom Inventory modified for the eye; **OR** = odds ratio; **OSDI** = Ocular Surface Disease Index; ***REEP5*** = receptor expression-enhancing protein-5; **SNP** = single nucleotide polymorphism; **SRP19** = *signal recognition particle-19*; **TBUT** = tear breakup time; ***ZRSR1*** = zinc finger RNA-binding motif and serine/arginine rich-1.

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