Research Paper

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The dichotomous role of epiregulin in pain

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Abstract

It has recently been shown that epidermal growth factor receptor (EGFR) contributes to the pathogenesis of pain. We scanned genetic markers within genes coding for receptors of the EGFR family (EGFR, ERBB2, ERBB3, and ERBB4) and their ligands (AREG, BTC, EGF, EPGN, EREG, HBEGF, MUC4, NRG1, NRG2, NRG3, NRG4, and TGFA) for association with self-reported pain intensity in patients with chronic facial pain who participated in the Orofacial Pain: Prospective Evaluation and Risk Assessment (OPPERA) cohort. We found that only epiregulin *(EREG)* was associated with pain. The strongest effect was observed for a minor allele at rs6836436 in *EREG*, which was associated with lower chronic pain intensity. However, the same allele was associated with higher facial pain intensity among cases with recent onset of facial pain. Similar trends were observed in an independent cohort of UK Biobank (UKB) where the minor allele at rs6836436 was associated with a higher number of acute pain sites but a lower number of chronic pain sites. Expression quantitative trait loci analyses established rs6836436 as a loss-of-function variant of EREG. Finally, we investigated the functional role of EREG using mouse models of chronic and acute pain. Injecting mice with an EREG monoclonal antibody reversed established mechanosensitivity in the complete Freund's adjuvant and spared nerve injury models of chronic pain. However, the EREG monoclonal antibody prolonged allodynia when administered during the development of complete Freund's adjuvant–induced mechanosensitivity and enhanced pain behavior in the capsaicin model of acute pain.

Keywords: EREG, SNPs, Genetic association studies, Hyperalgesia, Temporomandibular joint disorders, HER, EGFR, UK Biobank, OPPERA, CFA, Capsaicin, Pain

1. Introduction

Establishment of chronic pain is often a result of the body's inability to restore physiological homeostasis after acute pain.[24] Both acute and chronic pain states have large genetic component, which we have now started to identify.[31,51] The Orofacial Pain Prospective Evaluation and Risk Assessment (OPPERA) study was designed to examine and identify biopsychosocial, environmental, and genetic factors that contribute to the onset and chronicity of orofacial pain.[26] In the OPPERA cohort, case–control association analysis that focused on a common orofacial pain condition, temporomandibular disorders (TMDs), using a panel of 358 pain-relevant candidate genes, revealed that the genes encoding for the epidermal growth factor receptor (*EGFR*) and its ligand epiregulin (*EREG*) had the highest association with TMD risk.[30] However, genes of other EGFR ligands and receptors have not been tested for their association with human pain phenotypes.

Epiregulin, a member of the epidermal growth factor (EGF) family of peptide growth factors, plays important roles in angiogenesis and vascular remodeling. It is a potent mitogen with direct and indirect proinflammatory effects.[37] Epiregulin binds to EGFR (ErbB1) and ErbB4 (HER4) but also stimulates signaling of ErbB2 (HER2/Neu) and ErbB3 (HER3) through ligand-induced heterodimerization with a cognate receptor. Blocking EGFR with pharmacologically available small molecules and monoclonal antibodies produces analgesia in animals[30] and chronic pain patients.[19–21] For the current study, we hypothesized that the EREG–EGFR pathway uniquely contributes to the development and persistence of pain.

For the first time, we systematically screened single-nucleotide polymorphisms (SNPs) in all gene loci belonging to EGFR family receptors (namely, *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4*) and their ligands (namely, *AREG*, *BTC*, *EGF*, *EPGN*, *EREG*, *HBEGF*, *MUC4*, *NRG1*, *NRG2*, *NRG3*, *NRG4*, and *TGFA*) for their association with reported clinical pain in the OPPERA cohort. We chose to use characteristic pain intensity (CPI) as an outcome measure because of its clinical significance.[11] Our analysis indicated that from the 16 genes screened, only *EREG* gene SNPs were associated with CPI. Next, we characterized the association between *EREG* variants and other pain severity phenotypes, namely, acute pain intensity and the number of other chronic painful comorbidities in OPPERA. The same *EREG* variant that was protective for chronic pain intensity increased risk for acute pain intensity in OPPERA. We then validated the dichotomous effect of *EREG* using an independent cohort from the UK Biobank (UKB). We also demonstrated the direction of the genetic effect of the identified SNPs on corresponding mRNA

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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expression level through subsequent cis-expression quantitative trait loci (eQTL) analyses from 2 independent studies. Finally, the dichotomous role of EREG for pain phenotypes was tested using mouse models of chronic and acute pain sensitivity.

2. Methods

2.1. Cohort description

The OPPERA cohort was used as a discovery cohort for this study. The study methods have been described in detail elsewhere.[2] In summary, the prospective cohort study enrolled 3263 participants between May 2006 and November 2008 at 4 US study sites: Baltimore, Maryland; Buffalo, New York; Chapel Hill, North Carolina; and Gainesville, Florida, from which 3161 were genotyped. To be eligible for enrollment, the participants had to satisfy the selection criteria determined during telephone screening and at the baseline clinical visit. The facial pain characteristics were collected using the OPPERA Comprehensive Pain Symptom Questionnaire (CPSQ), and TMD was diagnosed by trained examiners using the Research Diagnostic Criteria for TMD (RDC/TMD).[10] Participants were followed at quarterly (3 monthly) intervals after the baseline visit with questionnaires and clinical visits. The project's protocol was approved by the institutional review boards at each OPPERA study site and at McGill University. Written informed consent was obtained from each participant before their enrollment.

To replicate our findings from OPPERA, data from UKB were used. Described in detail elsewhere,[44] the UKB study is a large prospective multicenter study of people living in the United Kingdom that had recruited 503,325 individuals between 2006 and 2010. Follow-up data were collected after 2012. Ethics approval for the UK Biobank study was obtained from the North West Centre for Research Ethics Committee (11/NW/0382), and all participants provided written informed consent. Their participation involved completing questionnaires, undergoing an interview with a trained nurse during which a range of physical measures was collected and donating samples of blood, urine, and saliva.

2.2. Outcome measures

The OPPERA study used the Research Diagnostic Criteria to define TMD cases.[10] According to this criteria, an individual was deemed as a chronic TMD case if an examiner had confirmed pain in the orofacial area for at least 5 days a month for ≥6 months and either ≥3 temporomandibular muscle groups or ≥1 temporomandibular joint painful to palpation or jaw movement. In addition, the OPPERA study used the CPSQ questionnaire, a self-report instrument, to assess the presence of multiple pain symptoms and associated characteristics.[40]

Comprehensive Pain Symptom Questionnaire asked the following screening question:

> *"Have you ever had pain in your face, jaw, temple, in front of the ear, or in the ear, not including toothache or ear infection?"*

If the participant answered "yes" to the aforementioned question, s/he was asked the following:

(1) *"How many years or months ago did your facial pain begin?"*
(2) *"How would you describe the duration of your facial pain?"*
(3) "*How would you rate your facial pain at the present time,* i.e., *right now?"*
(4) "*In the past 6 months, how intense was your worst facial pain?"*
(5) "*In the past 6 months, on average, how intense was your facial pain?"*

The responses of the above-mentioned question (1) were numerical (years and months) The responses to the question (2) were collected as either *"persistent," "recurrent,"* or *"one time,"* whereas, the responses to the questions (3) to (4) were collected using a numerical rating scale (NRS) where 0 was marked as "*no pain*" and 10 was "*pain as bad as it could be*." Measuring pain intensity on a NRS is a valid and clinically meaningful measure of pain severity.[13] Characteristic pain intensity is an arithmetic mean of the 3 NRS ratings, namely, pain right now, worst pain in 6 months, and average pain in 6 months. Contrary to NRS alone, CPI is temporally stable,[33] provides a more reliable estimation of pain severity,[11,47] and has been demonstrated to be a significant predictor of TMD chronicity.[12] Hence, we chose CPI as our primary outcome measure. Of 3161 genotyped OPPERA participants, 399 participants were excluded due to missing or poor-quality phenotype data. Of the remaining 2762 participants, 1626 never had pain in the facial region. Characteristic pain intensity scores were not calculated for these 1626 participants. Eight hundred ninety-four of the remaining 1136 participants had facial pain for more than 3 months. We restricted our analysis to the participants with either persistent (n = 124) or recurrent (n = 264) facial pain to comply with the latest definition of chronic pain according to the International Association for the Study of Pain (IASP).[45] The CPI scores of these 388 OPPERA participants at baseline were considered as the chronic pain intensity (**Fig. 1A**). Follow-up CPI scores of the controls with a CPI at baseline of zero were considered as an acute pain intensity marker (n = 213) (**Fig. 1A**), and participants with acute CPI >0 were considered as acute facial pain cases (n = 112). Other phenotypes from OPPERA included the number of comorbid pain conditions present from fibromyalgia, chronic fatigue syndrome, irritable bowel syndrome, interstitial cystitis, arthritis, and chronic pelvic pain, and TMD caseness, as described earlier.

As part of the UKB data collection framework, participants were asked: "*In the last month, have you experienced any of the following that interfered with your usual activities?*" (UKB data-field ID: 6159). Participants could choose all that apply from the following options: headache, facial pain, neck or shoulder pain, back pain, stomach or abdominal pain, hip pain, knee pain, pain all over the body, none of the above, and prefer not to answer. We generated a quantitative trait ranging from 0 to 8, corresponding to the number of sites reported as painful. Those reporting to have "*pain all over the body*" were assigned the maximum score of 8. If the site was painful for not more than a month, it was counted as an acute pain site. If a bodily site remained painful on follow-up after 2 or more years, then it was counted as a chronic pain site.

Caseness for participant's pain sites were established as follows in UKB (**Fig. 1B**):

(1) Participants who reported no pain sites at baseline and follow-up were treated as controls.
(2) If a participant reported pain at a particular body site for 1 month but not for 3 months, s/he was classified as an acute pain case for that particular site.
(3) If a participant reported pain at a particular site for 1 month at baseline and at the same site for more than 3 months at follow-up, s/he was classified as a chronic case for that particular site.

2.3. Genotyping

Peripheral venous whole blood was collected at each OPPERA site into 5-mL polyethylene tubes containing ethylenediaminetetraacetic acid (EDTA) (Vacutainer; Beckton Dickinson and Company, Franklin Lakes, NJ), and the tubes were stored in −80°C freezer.[41]

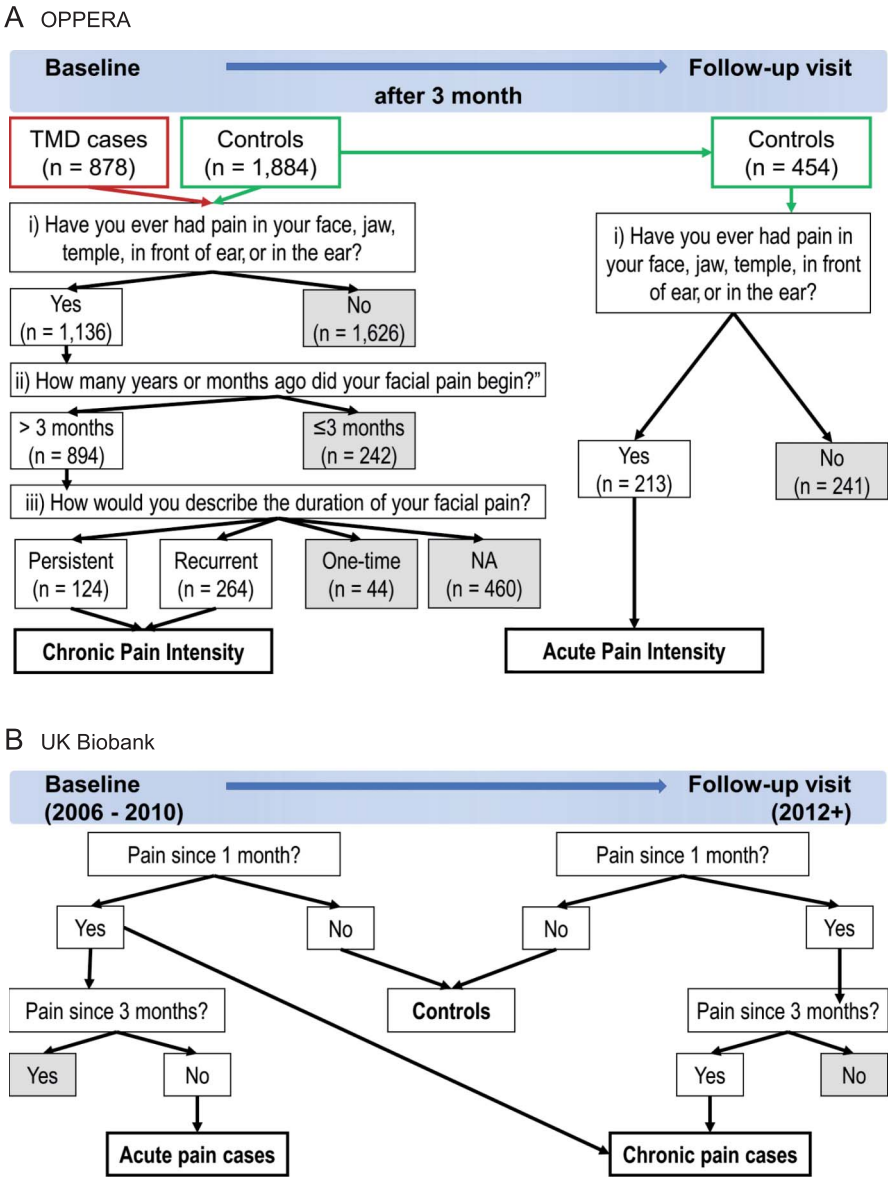

Figure 1. Selection of acute and chronic pain phenotypes in the (A) OPPERA and (B) UKB cohorts.

Genomic DNA was purified using the protocols of Qiagen extraction kits. Samples were genotyped using the Illumina HumanOmni2.5Exome-8v1A array (Illumina, Inc, San Diego, CA) at the Center for Inherited Disease Research (Johns Hopkins University, Baltimore, MD). The details of genotyping and QC procedures have been described elsewhere.[42] Genotyping results were returned for 3221 unique samples, representing the study participants. All the genotyped SNPs with minor allele frequency (MAF) greater than 5% in gene loci belonging either to EGF-family receptors (namely, *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4*) or ligands (namely, *AREG*, *BTC*, *EREG*, *EGF*, *EPGN*, *EREG*, *HBEGF*, *MUC4*, *NRG1*, *NRG2*, *NRG3*, *NRG4*, and *TGFA*) were chosen for the association analyses (n = 2407).

UKB's genetic data for 488,288 participants were used. As described in detail elsewhere,[8] blood samples were collected from participants on their visit to a UKB assessment center, and the samples were stored at the UKB facility in Stockport, UK. Over a period of 18 months, samples were retrieved, DNA was extracted, and shipped to Affymetrix Research Services Laboratory for genotyping. A subset of 49,940 participants was genotyped using the Applied Biosystems UK BiLEVE Axiom Array by Affymetrix (now part of Thermo Fisher Scientific).[48] Remaining 438,348 participants were genotyped using the closely related Applied Biosystems UK Biobank Axiom Array that shares 95% marker content with the UK BiLEVE Axiom Array. Routine quality checks were performed during the process of sample retrieval, DNA extraction, and genotype calling.

2.4. Mouse subjects

Male adult (7-9 weeks of age) CD1 [Crl:CD1 (ICR)] mice were acquired from Charles River Laboratories (Saint Constant, QC, Canada) and used for all experiments. All mice were housed in groups of 4 upon arrival, and procedures were conducted in accordance with the animal care standards set forth by the Canadian Council on Animal Care and were approved by the University of Toronto's Biosciences Panel on Laboratory Animal Care. All animals were maintained within

a temperature-controlled environment (23 ± 1°C) with a 12:12 h light:dark cycle. A compressed cotton nesting square and crinkled paper bedding were provided in each cage as a source of environmental enrichment. All mice had access to food (Harlan Teklad 8604) and water ad libitum.

2.5. Anti-EREG monoclonal antibody

A blocking/neutralizing EREG monoclonal antibody (mAb) (NBP2-21992; Novus Biologicals, Oakville, ON, Canada) was diluted in phosphate-buffered saline (PBS) and administered directly into the tail vein (5 μg/5 μL). Control mice were injected with an equivalent volume of PBS.

2.6. Mouse behavioral assays

2.6.1. von Frey tests

Mechanosensitivity was measured using the simplified up-down (SUDO) method with von Frey hairs to estimate the 50% withdrawal threshold in pressure units (g/mm^2).[7] Mice were placed on a perforated metal floor (with 5-mm-diameter holes placed 7 mm apart) within small Plexiglas cubicles (9 × 5 × 5-cm high), and a set of 8 calibrated von Frey fibers (Stoelting Touch Test Sensory Evaluator Kit no. 2 to no. 9; ranging from ~0.015 to ~1.3 g of force) was applied to the plantar surface of the hind paw until the fibers bowed and then held for 3 seconds. The threshold force required to elicit withdrawal of the paw (median 50% withdrawal) was determined twice on each hind paw (and averaged) for all measurements, with sequential measurements separated by at least 20 minutes.

2.6.2. Complete Freund's adjuvant

Complete Freund's adjuvant (CFA) (50%; Sigma-Aldrich, Oakville, ON, Canada) was injected intraplantar in a volume of 20 μL into the left hind paw using a 100-μL microsyringe with a 30-gauge needle. Mice were tested for mechanical thresholds of the injected hind paw using the von Frey test as described above, before, and at selected time points after CFA injection. The EREG mAb or vehicle control was injected 1 or 3 days after CFA injection.

2.6.3. Spared nerve injury

Spared nerve injury (SNI), an experimental nerve injury designed to produce neuropathic pain, was performed under isoflurane/oxygen anesthesia as described previously.[6,9] Briefly, using an operating microscope (X40), the 3 terminal branches of the sciatic nerve (tibial, sural, and common peroneal) were exposed. The tibial and common peroneal nerves were cut, after tight ligation with 6.0 silk, "sparing" the sural nerve. The incisions were closed in layers using interrupted sutures (6-0 Vicryl). Mice recovered on a heating pad—carefully monitored to prevent overheating—until ambulatory as per standard operating procedures. Mice were tested for mechanical sensitivity before and 14 days after surgery using the von Frey test as described above, except that the "spared" sural region was targeted for SNI by applying the fibers to the hind paw. After von Frey mechanical testing on day 14, mice were injected (intravenously) with the EREG mAb or vehicle control and then tested for mechanical sensitivity 16, 19, 21, and 23 days after surgery.

2.6.4. Capsaicin assay

Mice were placed on a tabletop within Plexiglas cylinders (30-cm high and 30-cm diameter) and allowed to habituate for 15 minutes. Mice then received a subcutaneous injection of capsaicin (2.5 μg; Sigma-Aldrich) into the plantar left hind paw (20 μL) and were digitally videotaped for 10 minutes. Video files were later scored for the total duration (s) of licking/biting (ie, nocifensive behavior) of the injected paw. Two hours after capsaicin behavior, mechanosensitivity was measured using the SUDO method (as described above). Care was taken to avoid the capsaicin injection site when testing mechanosensitivity. In these experiments, mice were pretreated with the EREG mAb or vehicle control 2 days before capsaicin injection. Withdrawal thresholds for the uninjected paw were also measured to determine whether the EREG mAb altered mechanical thresholds per se.

2.7. Antibody measurements

The concentration of the EREG mAb antibody conjugated to Alexa-647 (NBP2-21992AF647; Novus Biologicals) was determined using the Cytation 5 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT). In brief, either PBS or the EREG mAB-Alexa-647 was injected intravenously, and after 2, 5, or 7 days, mice were euthanized to collect blood. Blood was centrifuged at 5000 rpm for 20 minutes at 4°C to separate plasma and kept at −80°C until analysis. Plasma samples (100 μL/well) along with known standard concentrations of the EREG mAb were loaded into a 96-well microplate for fluorescence-based intensity measurement (Invitrogen, Burlington, ON, Canada). Using the multimode plate reader, fluorescence intensity of Alexa-647 was measured with a bandwidth of 20 nm (640-nm excitation and 681-nm emission). A standard curve was generated based on the fluorescence intensity values from the known standard concentrations, which was then used to calculate the concentration of the EREG mAB in the plasma samples.

2.8. Data analyses

The additive model of inheritance was assumed for all genetic analyses. The family-wise error rate was controlled using the Benjamini–Hochberg's false discovery rate (FDR) method[5] at 5% threshold. Pain phenotypes were considered as dependent variables, and minor allele counts of SNPs were independent variables for Poisson and logistic regression models for count and binary outcome measures, respectively. For initial screening of all the 2407 SNPs in EGFR family of receptors and ligands against chronic pain intensity, multivariate linear regression was conducted using PLINK (Broad Institute, Cambridge, MA), version 1.09.[35] Haplotype analyses were performed using Haplo.stats v1.7.7 (R-package),[23] which implements an expectation-maximization–derived score to test for a statistically significant association between haplotypes and outcome measurements. The statistical methods implemented in this R-package assume that all subjects are unrelated, and that haplotypes are ambiguous (due to unknown linkage phase of the genetic markers), while also allowing for missing alleles. Hence, unrelated participants from the OPPERA and UKB cohorts were selected using a second-degree relatedness threshold as implemented in Kinship-based INference for Genome-wide association studies (KING).[28] The effects of all rare haplotypes with the estimated frequency >5% in OPPERA and >2% in UK Biobank were compared against the effect of one ancestral haplotype. Generalized linear modelling was used to test for an association between genotypes and phenotypes. As the OPPERA participants were recruited in 4 study sites, recruitment sites were introduced as covariates in the regression models. Age, sex, and the first 3 principle components (the markers of ancestry) were

also included as covariates to adjust for population stratification. Similarly, the UKB data analyses were corrected for sex, age, ethnic background, and genotyping platforms. R v3.5.2 was used as the language and environment for statistical computation. Haplotype structure of *EREG* was analyzed using Haploview v 4.2.[4]

For eQTL analysis, 2 large-scale data sets, namely, the Framingham Heart Study (FHS)[18] and the Genotype-Tissue Expression (GTEx) project[14] (version 7), were used. The FHS data are available at dbGaP under the accession numbers phs000342 and phs000724. The GTEx data are also available at dbGAP under the accession number phs000424.v7.p2.

For mouse experiments, von Frey data were analyzed using repeated-measures analysis of variance (ANOVA), followed where appropriate by Tukey's honest significant difference post hoc test. Capsaicin nocifensive behavior was analyzed using independent *t*-tests.

3. Results

3.1. Among all EGFR receptors and ligands, only EREG was associated with pain

Our primary outcome measure, CPI, was well characterized in the OPPERA study. Hence, we used OPPERA as our discovery cohort. A total of 2407 genotyped SNPs with MAF >5% situated within the 16 genes of EGFR family receptors (ie, *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4*) and their ligands (*AREG*, *BTC*, *EGF*, *EPGN*, *EREG*, *HBEGF*, *MUC4*, *NRG1*, *NRG2*, *NRG3*, *NRG4*, and *TGFA*) were screened for an association with CPI in OPPERA (**Fig. 2**; supplementary Table 1, available at http://links.lww.com/PAIN/A937). Only 7 SNPs passed the FDR threshold of 5% after correcting for age, sex, recruitment site, and the first 3 principle components (**Table 1**). All significantly associated SNPs were located in the *EREG* gene with their minor alleles associated with less pain. Therefore, *EREG* was chosen as our primary candidate gene for further investigation.

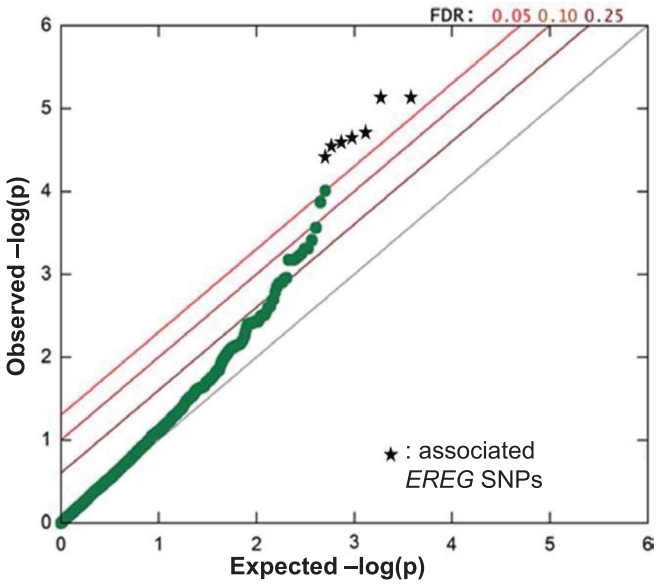

Figure 2. Quantile–quantile plot of 2407 SNPs in genes coding for EGFR superfamily receptors and ligands, showing a significant association (FDR < 0.05) between 7 *EREG* SNPs and chronic characteristic pain intensity (CPI) in OPPERA cohort. EGFR, epidermal growth factor receptor; EREG, epiregulin; FDR, false discovery rate.

3.2. EREG gene has 2 functional minor haplotypes

Of 2407 tested SNPs in the EGFR family of receptors and ligands, 7 SNPs within *EREG* were found to be significantly associated with CPI. The association results of the *EREG* SNPs were visualized along with their local linkage disequilibrium (LD), recombination patterns, and genomic region position. This regional plot of *EREG* (**Fig. 3A**) uncovered substantial LD structure between *EREG* SNPs, with one LD block within the gene (**Fig. 3B**; supplementary Table 2, available at http://links.lww.com/PAIN/A937). Furthermore, haplotype analysis identified 2 minor haplotypes, herein referred to as H2 and H3, with frequencies 17.3% and 5.8%, respectively, in OPPERA. All 7 significant SNPs were markers for the H3 haplotype of *EREG* (**Fig. 3C**), while we have previously reported the association of the H2 haplotype of *EREG*, marked by the functional SNP, rs2367707 with TMD,[30] and the H3 haplotype of *EREG* was not detected in our earlier analysis due to its relatively low frequency. A marker of the H3 haplotype, rs6836436, was deemed potentially functional because it was located in the 5′UTR region of *EREG*. Finally, the presence of reference allele (A) at rs1993665 exclusively marked the major haplotype (herein referred to as H1). Hence, rs1993665, rs6836436, and rs2367707 were chosen as the markers of H1, H2, and H3 haplotypes of *EREG* for haplotype association analyses in both OPPERA and UKB cohorts. Their minor allele counts and frequencies in OPPERA and UK Biobank are shown in **Table 2**. Haplotype frequencies based on the 3 marker SNPs in *EREG* as derived using expectation–maximization (E-M) algorithm were 67.33% and 74.65% for H1, 20.39% and 19.91% for H2, and 7.61% and 2.77% for H3 in OPPERA and UKB, respectively (**Table 3**). Expression quantitative trait loci databases, namely, FHS and GTEx, were scanned for the H2 (rs2367707) and H3 (rs6836436) SNP markers. Expression quantitative trait loci analyses revealed that both minor alleles at rs2367707 and rs6836436 were associated with decreased mRNA levels of *EREG* in the peripheral blood (**Table 4**).

3.3. H3 and H2 haplotypes of EREG protects from chronic pain

We hypothesized that genetic variability within the *EREG* locus may affect chronic pain intensity. From the OPPERA cohort, chronic pain intensity at baseline, TMD case status, and the number of chronic pain comorbidities were chosen as the chronic pain phenotypes for this study. Haplotype association analyses confirmed our previously reported protective role for the H2 haplotype for TMD case status (n = 2,755, OR = 0.84, FDR = 0.032, **Fig. 4A**).[30] Furthermore, the H3 haplotype of *EREG* was associated with lower chronic pain intensity (n = 388, $\beta = -8.06$, FDR = 0.033, **Fig. 4B**) and was marginally protective against the number of chronic pain comorbidities (n = 2,748, $\beta = -0.07$, FDR = 0.08).

For validation of these results, we used the UKB cohort. Pain intensity was not collected in the UKB cohort. Hence, we used the number of reported chronic pain body sites as a substitute for chronic pain intensity. Although pain severity in terms of intensity and anatomical extent are different phenotypes, they are correlated.[3,49] Painful sites were considered chronic if the pain persisted at the same site for at least 2 years, and the number of reported chronic pain body sites was used as a substitute for chronic pain intensity. In addition, the substantial size of the UKB cohort allowed us to consider pain at each of the 8 reported body

Table 1

Linear regression analyses of all the genes in EGFR receptor family and its ligands, and chronic characteristic pain intensity (CPI) in OPPERA cohort, corrected for age, sex, and the first 3 principal components.

Gene	SNP	Ref	Min	Base pair location	β	*P*-value	FDR
EREG	rs10518126	G	A	75,243,119	−11.09	0.0000078	0.00944**
EREG	rs57839099	G	A	75,243,813	−11.09	0.0000078	0.00944**
EREG	rs200889776	G	A	75,240,770	−10.41	0.00002	0.011*
EREG	rs57933408	G	A	75,243,828	−10.51	0.00002	0.011*
EREG	rs201835071	G	A	75,237,587	−9.99	0.00003	0.011*
EREG	rs72859363	A	G	75,246,112	−10.24	0.00003	0.011*
EREG	rs6836436	A	C	75,230,930	−8.84	0.00013	0.041*

FDR ≤ 0.01: "**" 0.05: "*". Only significant results (FDR ≤ 5%) are presented.
β, slope of least-squares line; EGFR, epidermal growth factor receptor; EREG, epiregulin; FDR, false discovery rate; Min, minor allele; Ref, reference allele; SNP, single-nucleotide polymorphism (rs ID).

sites as individual chronic pain phenotypes for this analysis. H2 was protective for the report of at least one chronic pain site (n = 196,534, OR = 0.95, FDR = 0.031, **Fig. 4C**) in UKB, whereas the presence of the H3 haplotype was associated with a decrease in the number of chronic pain sites (n = 196,534, β = −0.21, FDR = 0.003, **Fig. 4D**) and was protective for chronic hip pain (n = 191,669, OR = 0.66, FDR = 0.028). The results of all chronic pain phenotypes are summarized in **Table 5**. Together, both the H2

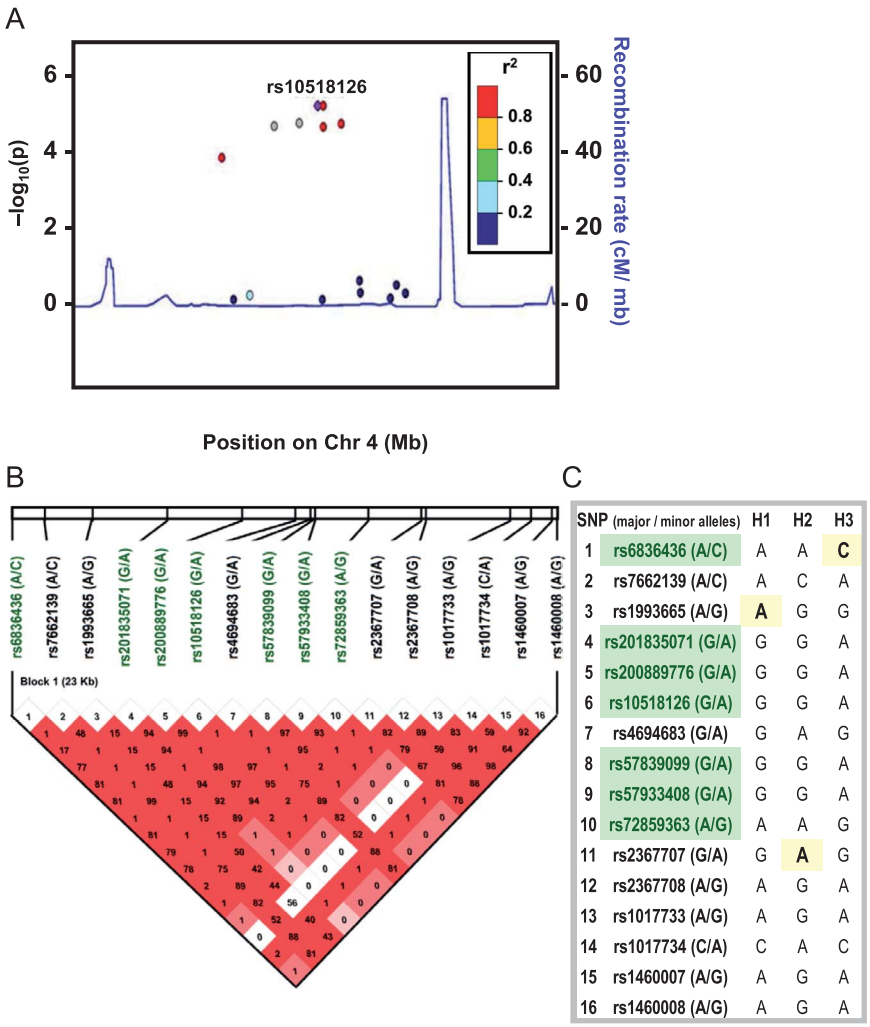

Figure 3. The *EREG* gene has 2 minor haplotypes. (A) Regional plot of *EREG*. (B) Illustration of the 16 SNPs in *EREG* linkage disequilibrium plot, numbers inside each cell indicate r^2 values, and color reflects D′ value, ranging from white to red, (ie, 0-1). (C) The sequence of 3 haplotypes with frequency >5% within *EREG* gene locus. Major and minor alleles of *EREG* SNPs genotyped in OPPERA, and SNPs significantly associated with CPI from Figure 2 are highlighted in green. Marker SNPs, namely, rs1993665, rs2367707, and rs6836436, for haplotypes H1, H2, and H3, respectively, are highlighted in yellow. CPI, characteristic pain intensity; EREG, epiregulin.

Table 2

Minor allele counts of the marker SNPs in OPPERA and UK Biobank cohorts.

MAC	Cohort	H1 marker SNP (rs1993665)		H2 marker SNP (rs2367707)		H3 marker SNP (rs6836436)	
		n	Frequency	n	Frequency	n	Frequency
0	OPPERA	1361	49.4%	1672	60.7%	2368	86.0%
1		1082	39.3%	956	34.7%	349	12.7%
2		312	11.3%	127	4.6%	38	1.4%
0	UK Biobank	267,521	58.5%	281,970	61.7%	432,835	94.7%
1		163,181	35.7%	154,109	33.7%	23,650	5.2%
2		26,568	5.8%	21,191	4.6%	785	0.2%

MAC, minor allele counts; n, number of participants.

and H3 haplotypes showed protective properties towards chronic pain with H3 haplotype displaying a stronger effect size consistent with its eQTL strength (defined by the slope, β, of the eQTL analysis, **Table 4**).

3.4. H3 haplotype of EREG is a risk for acute pain

Next, we tested the association of functional *EREG* haplotypes with acute clinical pain. To study the effects of minor haplotypes of *EREG* on acute pain, the first quarterly follow-up CPI scores measured in controls with baseline CPI = 0 were chosen as acute pain phenotype from the OPPERA cohort. Participants with no facial pain at baseline but CPI >0 after 3 months were considered acute facial pain cases (**Fig. 5A**). The number of reported acute (not more than 3 months) painful body sites was used as a marker of acute pain severity in UKB. In addition, participants with at least one reported acute painful site (n = 137,852) were contrasted against participants with no reported pain at all (n = 333,921). Haplotype H3 was strongly associated with acute pain but, unexpectedly, in the opposite direction compared with chronic pain. The presence of minor haplotype H3 was associated with an increase in acute pain intensity at follow-up (n = 213, β = 8.68, FDR = 0.039, **Fig. 5B**) in the OPPERA cohort. In the UKB, H3 was a risk factor for self-reported acute pain of at least one site (n = 471,773, OR = 1.34, FDR = 0.0002, **Fig. 5C**) and the total number of acute pain sites (n = 471,773, β = 0.028, FDR = 0.003, **Fig. 5D**). Moreover, haplotype H3 was associated with increased odds of having acute pain all over the body (n = 335,565, OR = 1.33, FDR = 0.0003). No significant association was detected between acute pain phenotypes and haplotype H2. The results of acute pain phenotypes are summarized in **Table 6**.

3.5. EREG has a dichotomous role in pain behavior in mice

Because our human genetic association results indicated that the H2 and H3 haplotypes may have a protective role against chronic pain and that these haplotypes were associated with lower *EREG* mRNA in the blood (**Table 4**), we hypothesized that blocking EREG would reduce pain hypersensitivity in mouse models of chronic pain. For these experiments, we blocked EREG by peripheral administration of an EREG mAb (5 μg) directly into the tail vein. Strikingly, mice injected with a single intravenous injection of the EREG mAb recovered quicker than control mice when the mAb was administered 3 days after CFA (ie, peak of allodynia) (two-way ANOVA, treatment × repeated measures: $F_{3,42} = 5.53$, $P = 0.003$, **Fig. 6A**). Since mice recovered from CFA-induced hypersensitivity by day 7 ($t_{14} = 1.35$, $P = 0.2$), it was difficult to determine whether the EREG mAb had long-lasting effects on allodynia or was specific for inflammatory pain. Thus, we assessed whether the EREG mAb reversed mechanosensitivity using the SNI model. For the SNI experiments, we administered the mAb 14 days after surgery to ensure that postoperative inflammation had resolved and that chronic mechanosensitivity had been established. As shown in **Figure 6B**, administration of the EREG mAb reversed mechanical hypersensitivity produced by SNI for up to 1 week after administration (two-way ANOVA, treatment × repeated measures: $F_{5, 84} = 10.49$, $P < 0.001$). In a subset of mice, we tracked the levels of the EREG mAb (conjugated to Alexa-647) by measuring fluorescence intensity in the blood plasma 2, 5, and 7 days after mAb administration. The concentration of the EREG mAb was significantly elevated at all time points after injection compared with control mice (two-way ANOVA, treatment × day after injection: $F_{2, 17} = 4.4$, $P = 0.027$, **Fig. 6C**).

Considering that the human genetic association results indicated that lost-function H2 and H3 haplotypes were a risk for acute pain, we further hypothesized that blockade of EREG during the development of pain states may prolong or enhance hypersensitivity. Administration of the EREG mAb 1 day after CFA (ie, during the development of hypersensitivity) delayed the natural recovery time course of mechanosensitivity (two-way ANOVA, treatment × repeated measures: $F_{2, 28} = 5.4$, $P = 0.001$, **Fig. 6D**). This effect seemed to be independent of an inflammatory state *per se*, as levels of white blood cells were not different in mice that received the EREG mAb or vehicle control (supplementary Table 3, available at http://links.lww.com/PAIN/A937). Next, we injected capsaicin as a model of acute pain (measuring both nocifensive and mechanical withdrawal thresholds). Pretreatment with the EREG mAb 2 days before capsaicin increased nocifensive behavior ($t_{14} = 2.54$, $P = 0.02$, **Fig. 6E**) and decreased mechanical pain thresholds in the injected, but not

Table 3

Haplotype frequencies of *EREG* as estimated through expectation–maximization (E-M) algorithm.

Haplotype*	OPPERA (n = 2755)	UK Biobank (n = 473,879)
H1	67.33%	74.65%
H2	20.39%	19.91%
H3	7.61%	2.77%
Log-likelihood	−4400.5	−563884.5
lr stat for no LD†	3404.9	588,766.6
P	8.63×10^{-08}	4.99×10^{-23}

* Haplotypes with frequencies > 5% in OPPERA and > 2% in UK Biobank.

† Likelihood ratio test statistic contrasting the log-likelihood for the estimated haplotype frequencies vs the log-likelihood under the null assuming that the alleles from all the 3 loci are in linkage equilibrium.

EREG, epiregulin; LD, linkage disequilibrium.

Table 4

Cis-eQTL in blood for the marker SNPs of minor haplotypes in *EREG*.

Cohort	n	SNP	Haplotype	β	*P*
FHS	2770	rs2367707	H2	−0.14	1.2×10^{-16}
		rs6836436*	H3	NA	NA
GTEx	369	rs2367707	H2	−0.09	2.4×10^{-02}
		rs6836436	H3	−0.27	1.4×10^{-04}

* H3 marker SNP, rs6836436, was not genotyped in Framingham Heart Study cohort.
eQTL, Expression quantitative trait loci; EREG, epiregulin; FHS, Framingham heart study; GTEx, Genotype-Tissue Expression project; n, number of participants; SNP, single-nucleotide polymorphism; β, slope of least-squares line.

uninjected paw (two-way ANOVA, treatment × paw: $F_{1, 28} = 4.91$, $P = 0.03$, **Fig. 6F**).

4. Discussion

In this study, we report the results of genetic screening within 16 genes of the EGFR family of receptors and ligands for their association with acute and chronic pain states. First, we identified *EREG* as the strongest sole contributor with 2 functional genetic variants and discovered a new haplotype H3 of *EREG* marked by the presence of a minor allele at SNP rs6836436. Second, and more surprisingly, we found that *EREG* has a dichotomous role in the pathophysiology of pain with its loss-of-function variants associated with decreased chronic pain severity but increased acute pain severity. We validated the results of this association analysis using mouse models of pain, where we found that neutralizing EREG with an mAb either reversed or enhanced pain behavior in chronic vs acute pain models, respectively.

Together, our results combined with previous reports[22,30] suggest that EREG mitigates pain during the early stages of its development but eventually contributes to the establishment of chronic pain. Therefore, in addition to current pharmacotherapy of chronic pain conditions with nonsteroidal anti-inflammatory drugs, opioids, corticosteroids, anxiolytics, muscle relaxants, antidepressants, anticonvulsants, and benzodiazepines, inhibition of EREG-EGFR complex formation could serve as a novel strategy to control chronic pain. Epiregulin-targeted therapy would not only be efficient in managing chronic pain but may provide a safer alternative to currently available drugs for EGFR

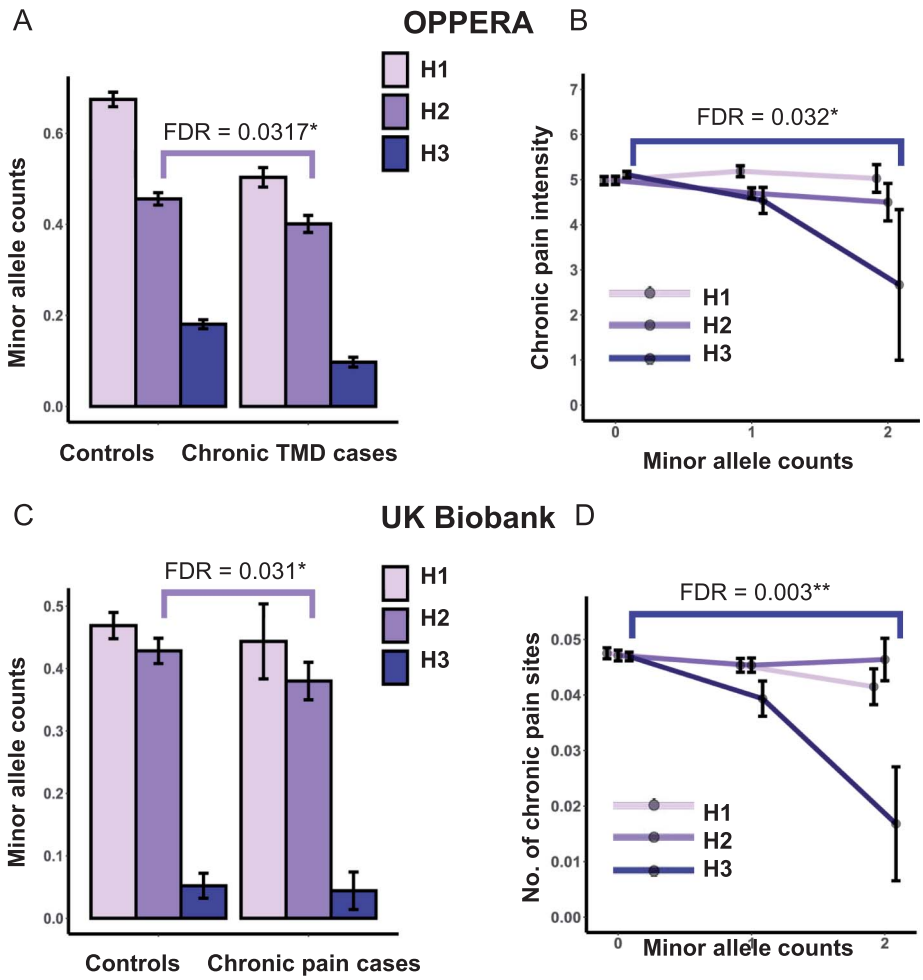

Figure 4. H3 and H2 haplotypes of *EREG* protects from chronic clinical pain. (A and B) OPPERA cohort. (A) Bar plot of average minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) among chronic TMD cases and controls and (B) plot of mean chronic pain intensity at baseline for minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) in the OPPERA cohort. (C and D) UKB cohort. (C) Bar plot of average minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) among chronic pain cases (at least one chronic pain site) and controls and (D) plot of mean number of chronic pain sites for minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) in the UKB cohort. Symbols represent mean ± SEM; false discovery rates (FDRs) were derived by generalized linear modelling for haplotype association; *FDR < 0.05; **FDR < 0.01. EREG, epiregulin; TMD, temporomandibular disorders.

Table 5

Haplotype association to analyze the relationship between chronic pain phenotypes and *EREG* haplotypes.

Cohort	Chronic pain phenotype	n	Prevalence	Haplotype II		Haplotype III	
				Estimate	FDR	Estimate	FDR
OPPERA	Chronic pain intensity	388	88.66%†	β = −3.11	0.245	β = −8.06	0.033*
	TMD case status	2755	31.87%	OR = 0.84	0.032*	OR = 0.79	0.083
	No. of chronic pain comorbidities	2748	78.93%‡	β = −0.04	0.174	β = −0.07	0.080
UK Biobank	Chronic pain all over the body	190,866	0.05%	OR = 1.08	0.660	OR = 0.95	0.913
	Chronic stomach or abdominal pain	191,111	0.18%	OR = 1.09	0.340	OR = 0.50	0.055
	Chronic headache	191,859	0.56%	OR = 0.91	0.112	OR = 0.96	0.804
	Chronic neck or shoulder pain	192,507	0.90%	OR = 0.96	0.309	OR = 0.80	0.082
	Chronic back pain	192,922	1.11%	OR = 0.98	0.521	OR = 0.83	0.081
	Chronic hip pain	191,669	0.47%	OR = 0.98	0.700	OR = 0.66	0.028*
	Chronic knee pain	192,796	1.05%	OR = 0.94	0.138	OR = 0.99	0.966
	Chronic facial pain	190,832	0.03%	OR = 0.77	0.305	OR = 0.27	0.204
	No. of chronic pain sites	196,534	2.93%‡	β = −0.031	0.107	β = −0.15	0.003*
	At least one chronic pain site			OR = 0.95	0.031*	OR = 0.90	0.131

FDR < 0.001: "**" 0.05: "*."

† Prevalence of TMD cases among n with valid chronic pain intensity scores at baseline.

‡ Prevalence of at least one chronic pain comorbidity/chronic pain site among total number of participants.

β, slope of least-squares line; CPI, characteristic pain intensity; EREG, epiregulin; FDR, false discovery rate; n, valid number of participants; OR, odds ratio.

inhibition because EGFR inhibitors have side effects such as skin rash.[20] Nonetheless, more definitive studies such as functional assays for rs6836436, preclinical experiments to further explore the role of EREG in acute and chronic pain, and clinical trials for EREG inhibitors as analgesics are required to substantiate the role of EREG in the pathogenesis of pain.

Like other chronic diseases, early intervention is associated with better outcomes with chronic pain. Hence, there is a need to identify potential chronic pain patients during the acute stage for timely and optimal disease management. On one hand, a risk biomarker indicates the potential for developing a disease in an individual who does not currently have an identifiable clinical disease. Being associated with increased chronic pain severity and risk for developing chronic TMD, the presence of a major allele at rs6836436 or rs2367707 in acute pain patients might serve as a risk biomarker of chronic pain development. Nevertheless, it is important to recognize that pain is a highly polygenic trait, and the contribution of each allele to the appreciable minor allelic frequency is expected to be modest. That is, why we do not suggest rs6836436 or rs2367707 by themselves will act as sole predictors of pain states but could be useful inclusions into a screening panel of genetic markers for pain profiling. Conversely, a response biomarker could identify individuals who are more likely to experience a favorable or unfavorable effect from drug treatment. The current findings suggest that the presence of a major allele at rs6836436 or rs2367707 may serve as a favorable response biomarker for EREG-EGFR–based pharmacotherapy of chronic pain. Thus, studying *EREG* gene polymorphism could accelerate the development of personalized pain medicine.

Although we used a TMD-centric cohort (OPPERA) as a discovery cohort, our results do not suggest that the association between *EREG* and pain phenotypes is specific to TMD or orofacial pain. We identified an association between *EREG* SNPs and a number of chronic pain conditions, independent of body site. This suggests that EREG contributes to TMD through mechanisms overlapping with other chronic pain conditions.[27] Symptoms of chronic TMD such as generalized pain sensitivity, sleep, concentration difficulties, depression, bowel complaints, and headaches often overlap with those of more generalized chronic pain conditions such as fibromyalgia and chronic fatigue syndrome.[1] However, our conclusions may be limited based on the statistical power of the discovery cohort, OPPERA. With a TMD incidence rate of 8% and MAF of *EREG's* H2 and H3 haplotype at 17.3% and 5% in the OPPERA follow-up cohort, respectively, the data lacked sufficient power to analyze the association between *EREG* and the onset of TMD in OPPERA or other associated pain phenotypes. Moreover, because of limited acute CPI at follow-up data, we could not confirm whether captured patients would resolve TMD in the OPPERA cohort or whether they would remain chronic.

It is important to recognize the absence of a good replication cohort for our discovery findings. We used CPI as a pain intensity marker in OPPERA, but this phenotype is rarely collected in large community cohorts such as UKB. Thus, we used the number of painful sites as markers of acute and chronic pain severity in UKB. Although pain severity in terms of intensity and anatomical extent are correlated,[3,49] these are clearly different phenotypes with potentially overlapping pathophysiology. Furthermore, the original discovery cohort was TMD-centric, while the UKB subjects reported pain across all body sites. Here, we viewed orofacial pain as an idiopathic pain condition and assumed that EREG contributes to it through molecular mechanisms shared by other chronic pain conditions, as suggested in our previous work.[30] Overall, our second analysis in the UKB cohort was a validation of the primary findings from OPPERA rather than a true replication. It, however, unambiguously supported the dichotomous nature of *EREG*'s contribution to pain.

We previously showed that both *EGFR* and *EREG* displayed a genetic association with chronic TMD where *EREG* showed the strongest association.[30] The current study confirms the association of haplotype H2 with chronic TMD in a different subset of OPPERA subjects (**Table 5**) and also demonstrated that the same haplotype H2 was associated with the presence of at least one chronic pain site in UKB. Although haplotype H3 was not identified in our earlier studies—due to its low frequency—it has a stronger effect than H2, as is evident from the strength of associations with pain phenotypes (**Tables 5 and 6**) and eQTL analysis (**Table 4**). However, both haplotypes, H2 and H3, are loss-of-function variants. Although we do not know the exact molecular mechanisms through which haplotypes H2 and H3 control *EREG* mRNA levels, we have shown earlier that rs2367707 (marker of H2) reduces stability of the mRNA, and 5′UTR location of rs6836436 (marker of H3) suggests control of

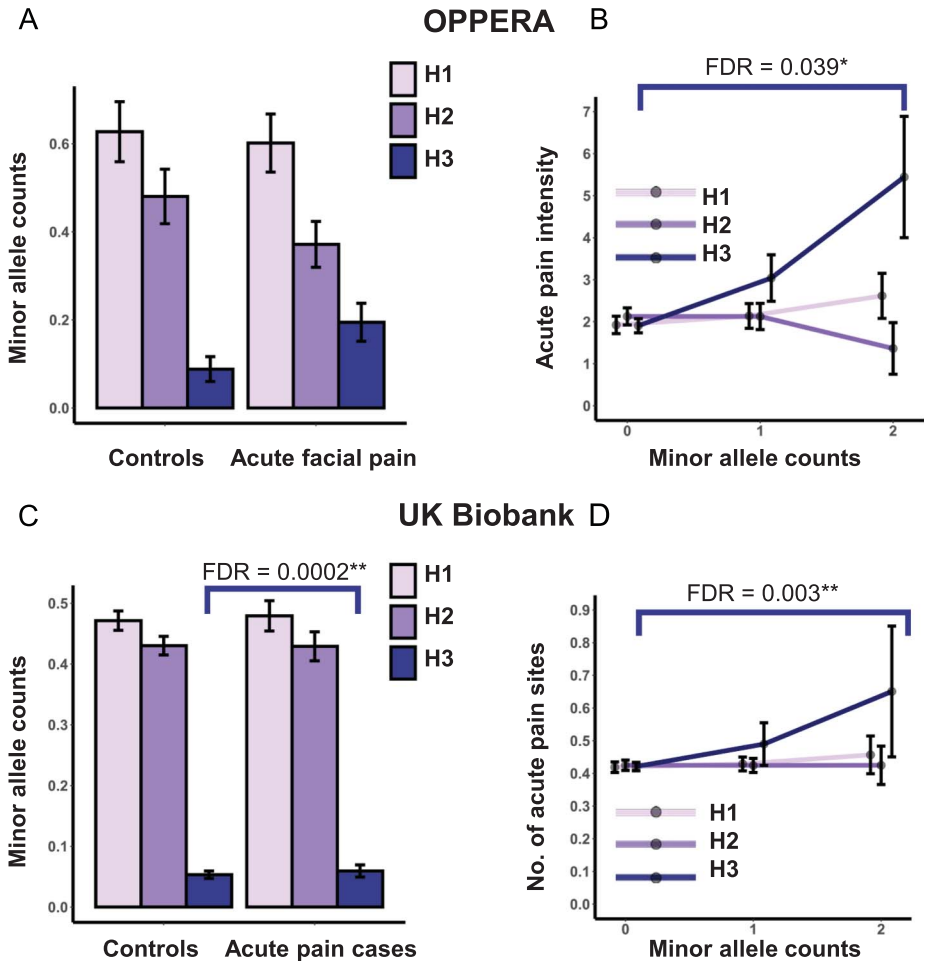

Figure 5. H3 haplotype of *EREG* is a risk for acute clinical pain. (A and B) OPPERA cohort. (A) Bar plot of average minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) among acute facial pain cases and controls and (B) plot of mean of acute pain intensity at follow-up in controls for minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) in the OPPERA cohort. (C and D) UKB cohort. (C) Bar plot of average minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) among acute pain cases (at least one acute pain site) and controls. (D) A plot of the mean number of acute pain sites for minor allele counts of rs1993665, rs2367707, and rs6836436, (markers for haplotypes H1, H2, and H3, respectively) in the UK Biobank (UKB) cohort. Symbols represent mean ± SEM; false discovery rates (FDRs) were derived by generalized linear modelling for haplotype association; *FDR < 0.05; **FDR < 0.01. EREG, epiregulin.

transcription. The absence of association of haplotype H2 with acute pain phenotypes (**Table 6**) is likely a reflection of its effect size rather than evidence for a unique contribution of H3, but not H2 to acute pain. An inverse relationship exists between the effect sizes and the allele frequencies for all phenotypic traits.[34] It is also possible that state-dependent stimuli regulating *EREG* transcription and mRNA stability contribute equally to chronic pain, but only regulation of transcription contributes to acute pain.

Our current results are also in line with our previous report on the effect of EREG in animal pain models. We previously showed that administration of EREG but not other EGFR ligands to mice in the late phase of the formalin test increased pain sensitivity.[30] Mouse experiments in this study assessed the impact of blocking EREG in different pain models at different time points and were designed to support and compliment the human genetic analysis. Mice treated with the EREG mAb during peak CFA allodynia (ie, 3 days after CFA) recovered quicker than control mice; SNI-induced allodynia was also reversed for up to 1 week after EREG mAb administration. These results provide generalizability across chronic pain assays and suggest that EREG neutralization may offer a novel analgesic strategy for established chronic pain. Furthermore, the EREG mAb delayed recovery from CFA when administered during the development of CFA-induced allodynia (ie, 1 day after CFA). EREG neutralization also enhanced nocifensive pain behavior and acute mechanosensitivity in mice injected with capsaicin. Broadly, these data support the findings from the human genetic analysis, where the H3 haplotype was found to be protective for chronic pain, but a risk marker for acute pain.

Although the signaling mechanisms of EREG on acute and chronic pain have yet to be discovered, a recently published independent study has reported similar dichotomous effects of EREG, where application of EREG onto the spinal dorsal nerve roots of rats reduced evoked c-fiber responses but increased spontaneous activity in spinal dorsal horn neurons.[22] Furthermore, immune system dysfunctions including allergic and autoimmune disease comorbidities[16,29,36,46] and elevated levels of proinflammatory cytokines[38,43,50] are common among chronic pain conditions. Because EREG is temporally[15] and causally[17,32] associated with activation of the immune system and inflammation,[37] EREG may contribute to pain through a systemic process. For instance, EREG is involved with the production of proinflammatory cytokines in macrophages,[39] and EREG is increased during cutaneous wound inflammation and healing.[25] Thus,

Table 6

Haplotype association to analyze the relationship between acute pain phenotypes and EREG haplotypes.

Cohort	Acute pain phenotype	n	Prevalence	Haplotype II		Haplotype III	
				Estimate	FDR	Estimate	FDR
OPPERA	Acute pain intensity	213	52.58%†	β = −1.83	0.517	β = 8.68	0.0390*
	Acute facial pain			OR = 0.76	0.289	OR = 1.99	0.121
UK Biobank	Acute pain all over the body	335,565	0.87%	OR = 1.01	0.909	OR = 1.33	0.0003**
	Acute stomach or abdominal pain	352,780	5.34%	OR = 0.99	0.261	OR = 0.94	0.066
	Acute headache	388,126	13.96%	OR = 0.99	0.246	OR = 1.00	0.771
	Acute neck or shoulder pain	369,264	9.57%	OR = 1.02	0.083	OR = 1.01	0.611
	Acute back pain	373,432	10.58%	OR = 0.99	0.672	OR = 0.98	0.447
	Acute hip pain	346,291	3.57%	OR = 0.99	0.820	OR = 0.98	0.758
	Acute knee pain	356,768	6.40%	OR = 1.00	0.810	OR = 1.03	0.408
	Acute facial pain	338,577	1.37%	OR = 0.97	0.405	OR = 0.95	0.541
	No. of acute pain sites	471,773	29.22%‡	β = −0.001	0.752	β = 0.028	0.003*
	At least one acute pain site			OR = 1.01	0.909	OR = 1.34	0.0002**

FDR < 0.001: "**" 0.05: "*."

† Prevalence of participants with acute pain intensity at follow-up > 0 among n with valid acute pain intensity scores at follow-up.

‡ Prevalence of at least one acute pain site.

β, slope of least-squares line; CPI, characteristic pain intensity; EREG, epiregulin; FDR, false discovery rate; n, valid number of participants/cases and controls; number of controls (no pain at all) is 333,936 for all the UK Biobank phenotypes; OR, odds ratio.

EREG production may be necessary for the resolution of inflammation and the natural recovery from pain; however, EREG may trigger expression of signaling cascades in primary afferent nerves or in the dorsal horn that promote long-term changes in neuronal excitability.[15,30,32] Nevertheless, a full understanding of the role of EREG in modulating pain severity through the immune and/or nervous system will require simultaneous study of EREG, immune cells, inflammation, and pain responses, both preclinically and clinically.

In conclusion, this study confirms the previously reported role of EREG in the pathogenesis of human chronic pain and preclinical pain models.[30] In addition, this study discovered an analgesic role for EREG during the early stages of pain, while an opposite-pronociceptive role in establishing chronic pain. Explicitly, this study is an example of a human → mouse translational research that further affirms EREG's potential as a biomarker of chronic pain, demystifies EREG-mediated pathogenesis of pain, and suggests a novel, nonopioid therapy for chronic pain.

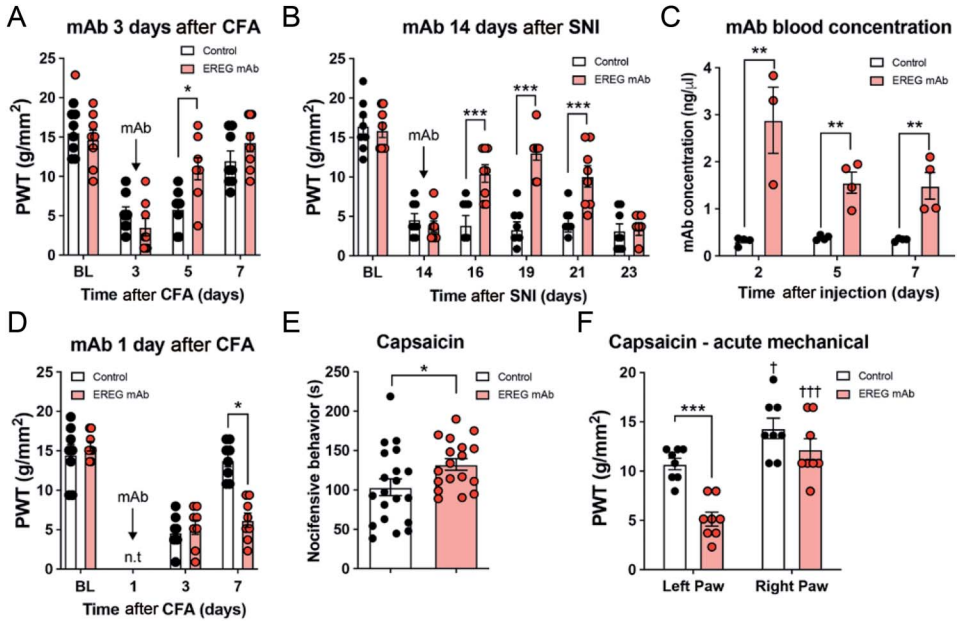

Figure 6. The effects of systemically administering an EREG monoclonal antibody (mAb, 5 μg) in mouse models of pain. (A) Mice injected with the EREG mAb 3 days after CFA, as indicated by the arrow have higher paw withdrawal thresholds (g/mm^2) compared with control mice 5 days after CFA; n = 8/group. (B) The EREG mAb or vehicle control was administered 14 days after SNI surgery, as indicated by the arrow. A single administration of the mAb reverses mechanical allodynia for up to 1 week. (C) The concentration of EREG mAb in the blood plasma of mice after a single tail vein administration; n = 4/group. (D) Mice injected with the EREG mAb 1 day after CFA, as indicated by the arrow have lower paw withdrawal thresholds (g/mm^2) than control mice 7 days after CFA; n = 8/group. (E) Pretreatment with the EREG mAb, 2 days before testing increases nocifensive behavior in the intraplantar capsaicin test of acute pain; n = 18 to 20/group. (F) A subset of mice from E was tested for mechanosensitivity after intraplantar capsaicin injection; n = 8/group. Mice injected with the EREG mAb have lower paw withdrawal thresholds (g/mm^2) in the capsaicin injected paw, but not the uninjected paw when compared with controls. BL: baseline.*$P < 0.05$; **$P < 0.001$; ***$P < 0.001$ compared with vehicle at the indicated time points. †$P < 0.05$; ††$P < 0.001$ compared with capsaicin-injected paw in F. CFA, complete Freund's adjuvant; EREG, epiregulin.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A937.

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References

[1] Aaron LA, Burke MM, Buchwald D. Overlapping conditions among patients with chronic fatigue syndrome, fibromyalgia, and temporomandibular disorder. Arch Intern Med 2000;160:221–7.
[2] Bair E, Brownstein NC, Ohrbach R, Greenspan JD, Dubner R, Fillingim RB, Maixner W, Smith SB, Diatchenko L, Gonzalez Y, Gordon SM, Lim PF, Ribeiro-Dasilva M, Dampier D, Knott C, Slade GD. Study protocol, sample characteristics, and loss to follow-up: the OPPERA prospective cohort study. J Pain 2013;14(12 suppl):T2–19.
[3] Barbero M, Fernandez-de-Las-Penas C, Palacios-Cena M, Cescon C, Falla D. Pain extent is associated with pain intensity but not with widespread pressure or thermal pain sensitivity in women with fibromyalgia syndrome. Clin Rheumatol 2017;36:1427–32.
[4] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263–5.
[5] Benjamini Y, Hochberg Y. Controlling the false discovery rate—a practical and powerful approach to multiple testing. J R Stat Soc B 1995;57: 289–300.
[6] Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. PAIN 1988;33: 87–107.
[7] Bonin RP, Bories C, De Koninck Y. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Mol Pain 2014;10:26.
[8] Bycroft C, Freeman C, Petkova D, Band G, Elliott LT, Sharp K, Motyer A, Vukcevic D, Delaneau O, O'Connell J, Cortes A, Welsh S, Young A, Effingham M, McVean G, Leslie S, Allen N, Donnelly P, Marchini J. The UK Biobank resource with deep phenotyping and genomic data. Nature 2018;562:203–9.
[9] Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. PAIN 2000;87:149–58.
[10] Dworkin SF, LeResche L. Research diagnostic criteria for temporomandibular disorders: review, criteria, examinations and specifications, critique. J Craniomandib Disord 1992;6:301–55.
[11] Dworkin SF, Von Korff M, Whitney CW, Le Resche L, Dicker BG, Barlow W. Measurement of characteristic pain intensity in field research. PAIN 1990;41:S290.
[12] Epker J, Gatchel RJ, Ellis E III. A model for predicting chronic TMD: practical application in clinical settings. J Am Dent Assoc 1999;130: 1470–5.
[13] Farrar JT, Young JP Jr, LaMoreaux L, Werth JL, Poole RM. Clinical importance of changes in chronic pain intensity measured on an 11-point numerical pain rating scale. PAIN 2001;94:149–58.
[14] GTEx_Consortium. The genotype-tissue expression (GTEx) project. Nat Genet 2013;45:580–5.
[15] Harada M, Kamimura D, Arima Y, Kohsaka H, Nakatsuji Y, Nishida M, Atsumi T, Meng J, Bando H, Singh R, Sabharwal L, Jiang JJ, Kumai N, Miyasaka N, Sakoda S, Yamauchi-Takihara K, Ogura H, Hirano T, Murakami M. Temporal expression of growth factors triggered by epiregulin regulates inflammation development. J Immunol 2015;194: 1039–46.
[16] Hoffmann RG, Kotchen JM, Kotchen TA, Cowley T, Dasgupta M, Cowley AW Jr. Temporomandibular disorders and associated clinical comorbidities. Clin J Pain 2011;27:268–74.
[17] Homma T, Kato A, Sakashita M, Takabayashi T, Norton JE, Suh LA, Carter RG, Harris KE, Peters AT, Grammer LC, Min JY, Shintani-Smith S, Tan BK, Welch K, Conley DB, Kern RC, Schleimer RP. Potential involvement of the epidermal growth factor receptor ligand epiregulin and matrix metalloproteinase-1 in pathogenesis of chronic rhinosinusitis. Am J Respir Cell Mol Biol 2017;57:334–45.
[18] Joehanes R, Zhang X, Huan T, Yao C, Ying SX, Nguyen QT, Demirkale CY, Feolo ML, Sharopova NR, Sturcke A, Schaffer AA, Heard-Costa N, Chen H, Liu PC, Wang R, Woodhouse KA, Tanriverdi K, Freedman JE, Raghavachari N, Dupuis J, Johnson AD, O'Donnell CJ, Levy D, Munson PJ. Integrated genome-wide analysis of expression quantitative trait loci aids interpretation of genomic association studies. Genome Biol 2017;18:16.
[19] Kersten C, Cameron MG. Cetuximab alleviates neuropathic pain despite tumour progression. BMJ Case Rep 2012;2012:bcr1220115374.
[20] Kersten C, Cameron MG, Laird B, Mjaland S. Epidermal growth factor receptor—inhibition (EGFR-I) in the treatment of neuropathic pain. Br J Anaesth 2015;115:761–7.
[21] Kersten C, Cameron MG, Mjåland S. Epithelial growth factor receptor (EGFR)-inhibition for relief of neuropathic pain—a case series. Scand J Pain 2013;4:3–7.
[22] Kongstorp M, Schjølberg T, Jacobsen DP, Haugen F, Gjerstad J. Epiregulin is released from intervertebral disks and induces spontaneous activity in pain pathways. PAIN Rep 2019;4:e718.
[23] Lake SL, Lyon H, Tantisira K, Silverman EK, Weiss ST, Laird NM, Schaid DJ. Estimation and tests of haplotype-environment interaction when linkage phase is ambiguous. Hum Hered 2003;55:56–65.
[24] Loeser JD, Melzack R. Pain: an overview. Lancet 1999;353:1607–9.
[25] Magnusson JE, Fisher K. The involvement of dopamine in nociception: the role of D(1) and D(2) receptors in the dorsolateral striatum. Brain Res 2000;855:260–6.
[26] Maixner W, Diatchenko L, Dubner R, Fillingim RB, Greenspan JD, Knott C, Ohrbach R, Weir B, Slade GD. Orofacial pain prospective evaluation and risk assessment study—the OPPERA study. J Pain 2011;12(11 suppl):T4–11.e12.
[27] Maixner W, Fillingim RB, Williams DA, Smith SB, Slade GD. Overlapping chronic pain conditions: implications for diagnosis and classification. J Pain 2016;17(9 suppl):T93–107.
[28] Manichaikul A, Mychaleckyj JC, Rich SS, Daly K, Sale M, Chen WM. Robust relationship inference in genome-wide association studies. Bioinformatics 2010;26:2867–73.
[29] Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. Nat Rev Neurosci 2005;6:521–32.
[30] Martin LJ, Smith SB, Khoutorsky A, Magnussen CA, Samoshkin A, Sorge RE, Cho C, Yosefpour N, Sivaselvachandran S, Tohyama S, Cole T, Khuong TM, Mir E, Gibson DG, Wieskopf JS, Sotocinal SG, Austin JS, Meloto CB, Gitt JH, Gkogkas C, Sonenberg N, Greenspan JD, Fillingim RB, Ohrbach R, Slade GD, Knott C, Dubner R, Nackley AG, Ribeiro-da-Silva A, Neely GG, Maixner W, Zaykin DV, Mogil JS, Diatchenko L. Epiregulin and EGFR interactions are involved in pain processing. J Clin Invest 2017;127:3353–66.
[31] Meloto CB, Benavides R, Lichtenwalter RN, Wen X, Tugarinov N, Zorina-Lichtenwalter K, Chabot-Dore AJ, Piltonen MH, Cattaneo S, Verma V, Klares R III, Khoury S, Parisien M, Diatchenko L. Human pain genetics database: a resource dedicated to human pain genetics research. PAIN 2018;159:749–63.

[32] Murakami M, Harada M, Kamimura D, Ogura H, Okuyama Y, Kumai N, Okuyama A, Singh R, Jiang JJ, Atsumi T, Shiraya S, Nakatsuji Y, Kinoshita M, Kohsaka H, Nishida M, Sakoda S, Miyasaka N, Yamauchi-Takihara K, Hirano T. Disease-association analysis of an inflammation-related feedback loop. Cell Rep 2013;3:946–59.
[33] Ohrbach R, Turner JA, Sherman JJ, Mancl LA, Truelove EL, Schiffman EL, Dworkin SF. The research diagnostic criteria for temporomandibular disorders. IV: evaluation of psychometric properties of the Axis II measures. J Orofac Pain 2010;24:48–62.
[34] Park JH, Gail MH, Weinberg CR, Carroll RJ, Chung CC, Wang Z, Chanock SJ, Fraumeni JF Jr, Chatterjee N. Distribution of allele frequencies and effect sizes and their interrelationships for common genetic susceptibility variants. Proc Natl Acad Sci U S A 2011;108:18026–31.
[35] Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559–75.
[36] Ren K, Dubner R. Interactions between the immune and nervous systems in pain. Nat Med 2010;16:1267–76.
[37] Riese DJ II, Cullum RL. Epiregulin: roles in normal physiology and cancer. Semin Cell Dev Biol 2014;28:49–56.
[38] Rodriguez-Pintó I, Agmon-Levin N, Howard A, Shoenfeld Y. Fibromyalgia and cytokines. Immunol Lett 2014;161:200–3.
[39] Shirasawa S, Sugiyama S, Baba I, Inokuchi J, Sekine S, Ogino K, Kawamura Y, Dohi T, Fujimoto M, Sasazuki T. Dermatitis due to epiregulin deficiency and a critical role of epiregulin in immune-related responses of keratinocyte and macrophage. Proc Natl Acad Sci USA 2004;101: 13921–6.
[40] Slade GD, Bair E, By K, Mulkey F, Baraian C, Rothwell R, Reynolds M, Miller V, Gonzalez Y, Gordon S, Ribeiro-Dasilva M, Lim PF, Greenspan JD, Dubner R, Fillingim RB, Diatchenko L, Maixner W, Dampier D, Knott C, Ohrbach R. Study methods, recruitment, sociodemographic findings, and demographic representativeness in the OPPERA study. J Pain 2011; 12(11 suppl):T12–26.
[41] Smith SB, Maixner DW, Greenspan JD, Dubner R, Fillingim RB, Ohrbach R, Knott C, Slade GD, Bair E, Gibson DG, Zaykin DV, Weir BS, Maixner W, Diatchenko L. Potential genetic risk factors for chronic TMD: genetic associations from the OPPERA case control study. J Pain 2011;12(11 suppl):T92–101.
[42] Smith SB, Parisien M, Bair E, Belfer I, Chabot-Dore AJ, Gris P, Khoury S, Tansley S, Torosyan Y, Zaykin DV, Bernhardt O, de Oliveira Serrano P, Gracely RH, Jain D, Jarvelin MR, Kaste LM, Kerr KF, Kocher T, Lahdesmaki R, Laniado N, Laurie CC, Laurie CA, Mannikko M, Meloto CB, Nackley AG, Nelson SC, Pesonen P, Ribeiro-Dasilva MC, Rizzatti-Barbosa CM, Sanders AE, Schwahn C, Sipila K, Sofer T, Teumer A, Mogil JS, Fillingim RB, Greenspan JD, Ohrbach R, Slade GD, Maixner W, Diatchenko L. Genome-wide association reveals contribution of MRAS to painful temporomandibular disorder in males. PAIN 2019;160:579–91.
[43] Sommer C, Kress M. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci Lett 2004;361:184–7.
[44] Sudlow C, Gallacher J, Allen N, Beral V, Burton P, Danesh J, Downey P, Elliott P, Green J, Landray M, Liu B, Matthews P, Ong G, Pell J, Silman A, Young A, Sprosen T, Peakman T, Collins R. UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age. PLoS Med 2015;12:e1001779.
[45] Treede RD, Rief W, Barke A, Aziz Q, Bennett MI, Benoliel R, Cohen M, Evers S, Finnerup NB, First MB, Giamberardino MA, Kaasa S, Korwisi B, Kosek E, Lavand'homme P, Nicholas M, Perrot S, Scholz J, Schug S, Smith BH, Svensson P, Vlaeyen JWS, Wang SJ. Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). PAIN 2019;160:19–27.
[46] Verma V, Sheikh Z, Ahmed AS. Nociception and role of immune system in pain. Acta Neurol Belg 2015;115:213–20.
[47] Von Korff M, Ormel J, Keefe FJ, Dworkin SF. Grading the severity of chronic pain. PAIN 1992;50:133–49.
[48] Wain LV, Shrine N, Miller S, Jackson VE, Ntalla I, Soler Artigas M, Billington CK, Kheirallah AK, Allen R, Cook JP, Probert K, Obeidat M, Bosse Y, Hao K, Postma DS, Pare PD, Ramasamy A, Consortium UKBE, Magi R, Mihailov E, Reinmaa E, Melen E, O'Connell J, Frangou E, Delaneau O, Ox GSKC, Freeman C, Petkova D, McCarthy M, Sayers I, Deloukas P, Hubbard R, Pavord I, Hansell AL, Thomson NC, Zeggini E, Morris AP, Marchini J, Strachan DP, Tobin MD, Hall IP. Novel insights into the genetics of smoking behaviour, lung function, and chronic obstructive pulmonary disease (UK BiLEVE): a genetic association study in UK Biobank. Lancet Respir Med 2015;3:769–81.
[49] Wolfe F. Pain extent and diagnosis: development and validation of the regional pain scale in 12,799 patients with rheumatic disease. J Rheumatol 2003;30:369–78.
[50] Zhang JM, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin 2007;45:27–37.
[51] Zorina-Lichtenwalter K, Meloto CB, Khoury S, Diatchenko L. Genetic predictors of human chronic pain conditions. Neuroscience 2016;338: 36–62.