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Pain Sensitivity and Opioid Analgesia: A Pharmacogenomic Twin Study

Martin S. Angst¹, Nicholas G. Phillips¹, David R. Drover¹, Martha Tingle¹, Amrita Ray², Gary E. Swan³, Laura C. Lazzeroni⁴, and J. David Clark^{1,5}

¹Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305, USA

²Departments of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA 94305, USA

³Center for Health Sciences, SRI International, Menlo Park, California 94025, USA

⁴Departments of Psychiatry and Behavioral Sciences and of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305, USA

⁵Department of Anesthesia, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA 94304, USA

Abstract

Opioids are the cornerstone medication for the management of moderate to severe pain. Unfortunately, vast inter-individual differences in dose requirements complicate their effective and safe clinical use. Mechanisms underlying such differences are incompletely understood, are likely multifactorial, and include genetic and environmental contributions. While accumulating evidence suggests that variants of several genes account for some of the observed response variance, the relative contribution of these factors remains unknown. This study used a twin paradigm to provide a global estimate of the genetic and environmental contributions to inter-individual differences in pain sensitivity and analgesic opioid effects. Eighty one monozygotic and 31 dizygotic twin pairs successfully underwent a computer-controlled infusion with the muopioid agonist alfentanil in a single occasion, randomized, double-blind and placebo-controlled study design. Pain sensitivity and analgesic effects were assessed with experimental heat and cold pressor pain models along with important covariates including demographic factors, depression, anxiety, and sleep quality. Significant heritability was detected for cold pressor pain tolerance and opioid-mediated elevations in heat and cold pressor pain thresholds. Genetic effects accounted for 12–60% of the observed response variance. Significant familial effects accounting for 24–32% of observed variance were detected for heat and cold pressor pain thresholds and opioid-mediated elevation in cold pressor pain tolerance. Significant covariates included age, gender, race, education, and anxiety. Results provide a strong rationale for more detailed molecular genetic studies to elucidate mechanisms underlying inter-individual differences in pain sensitivity and analgesic opioid responses. Such studies will require careful consideration of the studied pain phenotype.

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Corresponding address: Martin S. Angst, MD Department of Anesthesia, Stanford University School of Medicine 300 Pasteur Drive Stanford, CA 94305-5117 Phone/Fax: (650) 498-5109, (650) 725-8052 ang@stanford.edu.

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Introduction

Opioids are the cornerstone therapy for the control of moderate to severe pain. However, opioids are far from being ideal agents. One problematic feature is the enormous variation in dose requirements when treating patients with pain conditions including postoperative, cancer, osteoarthritic and low back pain [6; 13; 18; 34; 68; 71]. Empirically titrating opioids is an imperfect practice resulting in delayed analgesia and, potentially, overdose. Indeed, the rate of death from accidental overdose has increased exponentially as the practice of using opioids for the management of non-malignant pain has grown [88]. There are currently no validated criteria to estimate patient-specific opioid doses for any painful condition *a priori*. A refined understanding of the factors determining inter-individual differences in dose requirements would significantly advance the effective and safe clinical use of opioids.

Wide variations in opioid dose requirements are likely the result of genetic and environmental factors. Genetic factors include gene variants leading to alterations in the expression or function of their respective products, while environmental factors include social, cultural and other influences. In addition, differences in opioid dose requirements have been linked to specific covariates including age, gender and mood [15; 31; 39; 66]. While the impact of one or a few of these factors and covariates has been studied in multiple paradigms, estimates speaking towards the overall contribution of genetic and environmental effects to variations in opioid dose requirements are lacking.

The twin study paradigm is one of the few available designs providing global estimates of the genetic and environmental contributions to inter-individual differences [41; 69]. In fact, twin studies have examined inter-individual differences in pain sensitivity and results of these studies suggest that sensitivity to thermal, mechanical and chemical painful stimuli are all influenced by genetics [54; 59; 62]. These findings provide a foundation for corroborating reported associations between variants of several genes including COMT, DRD4, FAAH, KCNS, 1MC1R, OPRM1, SCN9A, and TRPA1, and differences in pain sensitivity [20; 24; 28; 37; 43; 57; 72].

The global genetic contribution to inter-individual variations in opioid dose requirements has not been studied systematically. While several gene association studies have linked variants of ABCB1, COMT, HT2A, 5-HTTLPR, KCNJ6, MC1R, and OPRM1 to differences in analgesic opioid effects in experimental and clinical settings, the fraction of these differences that is attributable to genetic effects has not been quantified [5; 11; 30; 47; 52; 57; 61]. However, global estimates of the genetic contribution to inter-individual differences in analgesic opioid responses are important to clarify whether genetic factors are of likely clinical importance, which would justify larger-scale and more detailed molecular genetic studies.

While the logistics and the safe conduct of interventional and quantitative pharmacological protocols in twins is demanding, the feasibility of such an approach has recently been demonstrated [3]. The objective of this study was to provide estimates of the genetic and environmental contribution to inter-individual differences in pain sensitivity and opioid analgesic effects in twins undergoing precisely controlled opioid infusions and quantitative experimental pain tests under laboratory-type conditions.

Methods

This pharmacogenomic study produced large data sets covering four outcome domains. Here we present data on pain sensitivity and analgesic opioid effects, while data on aversive and reinforcing opioid effects will be reported in a separate manuscript. The study was registered at ClinicalTrials.gov on May 2, 2008 (NCT00672438; PI: Angst MS). A detailed description

of the methods and procedures required for the conduct of an interventional and laboratory-type pharmacogenomic study in a sizable number of twins has previously been published [3].

Subjects

Twins were recruited by a joint effort of SRI International and Stanford University School of Medicine. Recruitment was mainly achieved through the Twin Research Registry and advertisements broadcasted by regional radio stations [78]. The study was approved by the Institutional Review Boards of SRI International and Stanford University School of Medicine. Two hundred and forty two monozygotic and dizygotic twins were enrolled after giving written informed consent. A medical history was taken and participants were screened for inclusion and exclusion criteria. Inclusion criteria were 1) age 18–70 years, 2) fluency in the English language, and 3) negative urine pregnancy test on the study day (pre-menopausal women). Exclusion criteria were 1) clinically relevant systemic diseases such as psychiatric, neurological, and dermatological conditions interfering with the collection and interpretation of study data, 2) cardio-respiratory diseases causing at least moderate impairment in daily activities, 3) renal and hepatic diseases with functional impairment, 4) morbid obesity, 5) sleep apnea, 6) history of addiction, 7) allergy to study medication, 8) chronic intake of medications with recognized analgesic/antihyperalgesic activity, 9) intake of over-the-counter analgesics within two days prior to the study, 10) Raynaud's disease, 11) pregnancy, and 12) other conditions compromising a participant's safety or the integrity of the study.

Study setting

The study took place in the Human Pain Laboratory of the Department of Anesthesia at Stanford University School of Medicine. The laboratory offers a quiet environment and precise lighting and temperature control. Critical equipment for the successful and safe conduct of the study included ergonomic and adjustable treatment chairs (Cloud 9, Living Earth Crafts, Vista, CA), vital signs monitors (Propaq Model 244, Welch Allyn, Beaverton, OR), 3) oxygen supply, and 4) a crash cart with a defibrillator, airway management equipment and emergency drugs. Laboratory staff included 1) a research associate who was blinded to treatment, solely interacted with study participants, performed all testing procedures, and collected all subjective and behavioral data, 2) a registered nurse trained in critical care or emergency medicine who was not blinded to treatment, performed the phlebotomy, administered the study drug, monitored and recorded vital signs, and collected blood specimens, and 3) an anesthesiologist who was blinded, and was physically present to oversee the drug infusion and assure the safety of study participants. Vital signs including heart rate (electrocardiogram), blood pressure, and hemoglobin oxygen saturation were monitored throughout the study. Participants were required to fast overnight except for clear liquids that were allowed up to 2 hours before starting the drug infusion. Subjects were also required to have at least 6 hours of night-time sleep before a study session. Twin pairs were not allowed to share their study experience before the completion of the experiments in both twins. During the drug infusion participants received supplemental oxygen (2l/min) via nasal cannula. Resting periods during the study were standardized, the room light was dimmed, subjects listened to relaxing music of their choice via headphones, and activities by study staff causing noise or possibly distracting subjects were prohibited. At the end of the study session subjects were discharged when they met criteria used for patients undergoing non-invasive, ambulatory procedures requiring sedation (e.g., colonoscopy). Criteria included 1) blood pressure $\pm 20\%$ of baseline, 2) hemoglobin oxygen saturation $>95\%$, 3) awake, 4) no or mild nausea, 5) no vomiting, 6) able to urinate, and 7) pre-arranged transport home available.

General study design and randomization

Twins underwent a single occasion, randomized, double-blind and placebo-controlled study protocol (Figure 1). During the pre-infusion phase pain sensitivity and covariates potentially affecting pain sensitivity were assessed. Blood was collected for genotyping. During the infusion phase changes in pain sensitivity were measured to infer analgesic drug effects and blood was collected to assay for drug plasma concentrations. Fifty percent of the twin pairs were randomized to receive an infusion of the mu-opioid agonist alfentanil followed by the infusion of saline placebo, while the other 50% of twin pairs were randomized to receive the infusions in reverse order. The randomization list allocating twin pairs to a particular infusion sequence was generated via Research Randomizer (<http://www.randomizer.org/>).

The single occasion study design had to control for placebo effects that could potentially confound measures of pain sensitivity and analgesia. Traditional study designs test for drug and placebo effects on separate study occasions. However, asking twins to return for a second study occasion was not considered feasible, since such a requirement may have hampered our ability to recruit and retain a sufficient number of twins. For example, 31% of twins lived more than 60 miles away from the study location. While a single occasion design did not allow assessing placebo effects in all participants, such effects could be assessed in the 50% of twins randomized to receive saline placebo before alfentanil. Randomizing the other 50% of twins to receive alfentanil before saline placebo was necessary to maintain the blinding. However, placebo effects could not be assessed in these twins because residual alfentanil plasma concentrations were still present during the saline placebo infusion.

Opioid administration and assay

A computer-controlled infusion (CCI) paradigm was used to quickly achieve and maintain steady-state drug plasma and effect site concentrations. An equilibration period of 20min was observed between starting the infusion of alfentanil or saline placebo and the first assessment of drug effects, which allowed measuring all outcomes at similar drug concentrations [4]. While a CCI allows maintaining a stable plasma concentration in an individual subject, plasma concentrations vary among participants. Therefore, alfentanil plasma concentrations were measured to include inter-individual differences in drug concentrations as a covariate in the final analysis. The μ -opioid agonist alfentanil (Janssen Pharmaceutica, Titusville, NJ) was chosen because of its quick onset and offset of action, and a previously validated CCI algorithm for its administration [4]. Alfentanil was administered intravenously via a computer-controlled infusion pump (Harvard Pump 22, Harvard Apparatus, Inc., South Natick, MA) targeting a steady-state plasma concentration of 100ng/ml. This target concentration was achieved in stepwise fashion over the course of 4min by targeting intermediate target concentrations of 30 and 70ng/ml. The target concentration of 100ng/ml produces clinically relevant analgesic effects in the average subject and significantly attenuates experimental pain without causing harmful side effects [4; 84]. STANPUMP using Scott's weight-adjusted pharmacokinetic parameters was the software driving the infusion pump [75].

Alfentanil target concentrations of 100ng/ml are associated with a modest increase in reaction time. A previous study reported an average increase of 20 and 86ms for target concentrations of 80 and 160ng/ml.[4] Therefore, sedative effects of alfentanil at a target concentration of 100ng/ml are not expected to significantly confound analgesic effect measures that dependent on reaction time (see below).

Alfentanil plasma concentrations were assayed at the Clinical Research and Development Unit of the Department of Anesthesia at the University of Colorado Health Sciences Center (Denver, CO). Six milliliters of venous blood were drawn into heparinized glass tubes,

centrifuged, and the plasma was frozen and stored at -70°C until assayed. Using LC/LC-MS/MS the lower limit of quantitation was 1.25 pg/ml with a 1000-fold linear range ($R=0.99$), and an intra- and between-assay coefficient of variations ranging between 4–16% and 3–14%.

Experimental pain tests

While experimental and clinical pain are not the same, experimental pain can mimic important mechanistic and phenomenological aspects of clinical pain and produce valid surrogate data [38; 67; 87]. Two mechanistically distinct experimental pain models were used. All subjects were trained on the study day to reliably perform in both experimental pain models before starting data collection. A reliable performance was defined as a difference smaller than 0.8°C for measures of heat pain sensitivity, and a difference smaller than 20% for measures of cold pressor pain sensitivity in two consecutive test sessions. Heat pain sensitivity was always determined before cold pressor pain sensitivity. A minimal resting period of 5min was strictly observed between subsequent test sessions.

Heat pain—Heat pain evoked by slowly raising temperatures is mediated by unmyelinated nociceptive fibers and is potentially affected by opioids [2; 89]. Heat pain was induced with a thermal sensory analyzer (TSA-II, Medoc Advanced Medical Systems, Durham, North Carolina). A $3\times 3\text{cm}$ thermode was placed in contact with skin at the volar forearm. Starting at 35°C , the thermode temperature was increased at a rate of 1°C/s . Study participants pushed a button of a hand-held device at the onset of pain. This procedure was repeated 4 times with an inter-stimulus interval of 30s. The average temperature eliciting pain was recorded as the pain threshold. The maximum thermode temperature was limited to 52°C to prevent burn injuries. The heat pain tolerance (maximum tolerated pain) was not assessed in this study. A relevant number of subjects would tolerate thermode temperatures greater than 52°C at baseline, and an even greater number would tolerate such temperatures during the infusion of alfentanil. The large resulting set of censored analgesic data would significantly limit the analysis of genetic and environmental effects.

Cold pressor pain—The cold pressor pain is thought to mimic important qualities of clinical pain, since verbal descriptors for both types of pain are strikingly similar [14]. Cold pressor pain is also mediated by small unmyelinated nociceptive fibers but is more sustained than heat pain, arises from deeper tissue structures and is associated with a much stronger affective response [46; 70]. The cold pressor pain model can be viewed as a tool examining an integrated pain response with a strong affective component, while the heat pain model is better suited to explore sensory-discriminative aspects of pain. Sensitivity to cold pressor pain was tested by asking subjects to immerse their hand up to the wrist in ice-water ($1\text{--}2^{\circ}\text{C}$) continuously re-circulated within a 12-liter container with the palm of the hand in full contact with the bottom of the container. Subjects were instructed to focus on dull, aching pain projected into the wrist or the palm, while ignoring accompanying sensations such as tingling, cold or numbness. They were asked to indicate the onset of pain and to withdraw the hand once pain became intolerable. The time to the onset of pain was recorded as the pain threshold and the time to withdrawing the hand was recorded as the cold pressor pain tolerance. The maximum exposure duration was limited to 180s.

Covariates

Several covariates potentially affecting measures of pain sensitivity and analgesia were assessed.

Demographics—The perception of pain can be affected by several demographic factors including race, ethnicity, gender, age, and educational status [1; 29; 32; 79; 85].

Anxiety—Anxiety correlates with the severity of pain [14; 80]. Anxiety was assessed with the Profile of Mood States (POMS), a self-reported questionnaire that evaluates six dimensions of mood (anxiety, depression, anger, vigor, inertia, and bewilderment)[55]. Subjects rated 65 mood-related adjectives on a 5-point scale (0 = not at all, 1 = a little, 2 = moderately, 3 = quite a bit, 4 = extremely). The POMS yields a total score and sub-scores for each dimension of mood (anxiety sub-score range: 0–36).

Depression—Depression is associated with more frequent and severe pain [51]. Depressive symptom severity was assessed with the Beck Depression Inventory (BDI), a self-reported questionnaire yielding a single score between 0–63 (0–13 = no depression, 14–19 = mild depression, 20–28 = moderate depression, >29 = severe depression) [8].

Sleep—Disturbed sleep is associated with increased pain sensitivity [65]. Sleep quality during the month preceding the study was assessed with the Pittsburgh Sleep Quality Index (PSQI), a self-reported questionnaire that assesses seven components of sleep (quality, latency, duration, efficiency, disturbance, medication, and daytime dysfunction). The total score ranges between 0–21, and a value >6 is indicative for sleep disturbance.

Blood pressure—Blood pressure and pain sensitivity have been found to be negatively correlated in some studies [10; 12]. Systolic and diastolic blood pressure was measured twice at each arm before drug exposure after observing a 15-minute resting period in semi-recumbent position (Propaq Model 244, Welch Allyn, Beaverton, OR).

Zygosity testing

Zygosity was assessed by genotyping forty seven single nucleotide polymorphisms [35]. Genotyping was performed with a custom-designed Oligo Pool for Methylation Assay (Golden Gate Genotyping Assay, Illumina Inc, San Diego, CA) and BeadXpress (iGenix Inc., Bainbridge Island, WA).

Statistical analysis

Data are presented as mean and standard deviation (SD) or as median and inter-quartile range (IQR). Summary statistics, parametric or non-parametric hypothesis testing with paired or non-paired test procedures on continuous and ranked data were performed in Systat Version 13 (Chicago, IL). Correlational analysis used cluster resampling techniques to account for interdependences within twin pairs (see below). An alpha level of $p < 0.05$ indicated statistical significance. Censored data were included in the analysis. Specifically, data from subjects reaching cut-off values when tested for heat and cold pressor pain sensitivity were included as the cut-off value. Analgesic effects to heat pain were inferred by subtracting the baseline heat pain threshold from the threshold obtained during the infusion of alfentanil. Analgesic effects to cold pressor pain were inferred by calculating the percentage increase of the baseline cold pressor pain threshold and tolerance during the infusion of alfentanil. Cold pressor pain data were log-transformed for analysis.

Statistical analyses of heritability (genetic effects), common environmental and familial effects (genetic and/or shared common environmental effects) was based on a generalized form of the Defries-Fulker (DF) regression model for twins [22]. In the DF model, phenotypic measurements of each twin are regressed on his or her co-twin's phenotypic measurement, while accounting for the joint distribution of the twin data. In contrast to alternative methods of twin analysis, the DF regression model produces unbiased estimates of twin pair intra-class correlations, which provide the basis for estimates of genetic and familial effects [48; 73]. The DF regression model was modified to allow for the simultaneous estimation of covariate effects, as well as genetic and environmental effects.

The method is equivalent to competing methods in terms of power but is more robust in the presence of non-normality. The modified model was specifically developed to address the highly skewed distribution of the visual analog scale data collected in of this study.

Simultaneous estimation of covariates as well as genetic and environmental effects was accomplished by concurrent fitting of two regression equations. The first equation reflects the original DF regression model using phenotypes adjusted for covariate effects. The second equation reflects a weighted regression model using phenotypes as the dependent variable and assorted covariates as the independent variables. Inverse variance weights were inferred from correlations determined with the first equation. In a few twin pairs, missing data caused one twin to be omitted from the analysis. In this case, the alternate twin contributed data to the fitting of the second but not the first regression equation.

We considered alternative analytical approaches using maximum likelihood methods (ML) in either structural equation or mixed effects models. However, these alternatives rely on normality assumptions and large sample-theory, which did not apply to several of our data sets as transformation to normality could not be achieved. The computational stability of the DF approach allowed the use of bootstrap approaches for estimating standard errors, confidence intervals, and p-values. The bootstrap requires repetition of the analysis in numerous random data sets. For ML and SEM, the bootstrap may fail to produce valid estimates on a sufficient number of random data sets. We conducted simulations to compare robustness and computational stability between the DF and ML approach.

Comparisons were based on 500 simulated data sets for each of twenty different scenarios. Scenarios varied in sample size (100 versus 400 twin pairs), the error distribution of the measured phenotype (normal versus exponential), and the established effect size as well as the genetic and environmental contributions to a phenotype (no, weak or strong effects; genetic and/or environmental effects). DF provided greater computational stability than ML, reaching convergence in 100% as opposed to 96% of all data sets. ML convergence rates varied across scenarios, with poorer performance for scenarios with weak or null effects. DF and ML were equivalent in precision (mean squared error of estimates), as each provided more precise estimates in about 50% of the tested scenarios. DF and ML showed similar test performance, although DF yielded slightly increased statistical power (1–7%). In the presence of non-normality when large-sample theory was used for both methods, DF was more robust than ML with false positive rates of 8 and 14%, respectively. The simulated 8% false positive rate under non-normality led us to combine the DF method with the bootstrap, which is a non-parametric method designed to increase robustness when model assumptions may be in doubt.

To identify the most significant covariate effects, a forward selection algorithm using the DF regression model with both genetic and environmental effects was applied to each of the studied phenotypes. Covariates were added sequentially, starting with the covariate associated with the smallest p-value ($p < 0.05$; Wald test for the corresponding Z-statistic). This procedure was repeated until remaining covariates no longer yielded a p-value < 0.05 .

All subsequent tests of genetic and familial effects incorporated the significant covariates for the respective phenotypes. Z-statistics (Wald test) were used for significance testing. Reported significance levels and 95% confidence intervals for selected covariates, and genetic and familial effects were estimated by generating 10,000 random bootstrap data sets. Data sets were created with clustered resampling techniques using twin pairs as the resampling unit. Bootstrap resampling was stratified by zygosity. A one-sided Wald test was used to test for a significant genetic effect. This test examined whether the intra-class correlation within monozygotic twin pairs was significantly larger than the intra-class

correlation for dizygotic twin pairs, using the standard assumption that monozygotic and dizygotic twins shared a common environment. If the genetic test was not statistically significant, a secondary one-sided test procedure was used to test for a significant familial effect. This test examined whether the average twin-pair intra-class correlation, assumed to be equal for monozygotic and dizygotic pairs, was greater than zero.

DF estimates of genetic and environmental effects were calculated under the ACE twin model that allows separate contributions of additive genetic (A), shared environmental (C), and unique environmental effects. Total familial effects were also estimated as A+C. For A, C, and A+C percentile bootstrap confidence intervals were used.

We conducted a secondary analysis using a fixed set of covariates to confirm the robustness of our primary forward selection covariate analysis. Fixed covariates were gender and age. For drug effects, fixed covariates also included drug plasma concentration and order of drug infusion. The fixed covariate analysis was based on the same computational principles outlined for the selected covariate approach.

Another secondary analysis was conducted in the largest homogeneous ethnic and racial subgroup (i.e., non-Hispanic Caucasians) to address the question as to whether results were significantly affected by the ethnic and racial heterogeneity of our study population.

Power analysis

The power analysis was based on the enrollment of 85 monozygotic and 40 dizygotic twin pairs. Power was approximated based on Fisher's log transformation for the Pearson correlation coefficient between measurements for twin A and twin B at an alpha level of $p < 0.05$. Eighty-five MZ pairs yielded a power of 0.8 to detect familial aggregation $> 30\%$. Eighty-five MZ and 40 DZ pairs yielded a power of 0.5 to detect heritability $> 50\%$ assuming that common environmental effects account for a modest 20% of the variance. However, these calculations were best approximations because of uncertainty regarding the relative contribution of additive genetic (A), shared environmental (C), and unique environmental (E) effects to the response variance.

Results

Subjects

A total of one hundred and twenty one twin pairs were recruited. Only twin pairs but no single twins were recruited. One hundred and fourteen pairs completed the study. Seven pairs were excluded for the following reasons: 1) two pairs because of positive pregnancy test, 2) two pairs due to missed appointments, and 3) three pairs showed poor compliance with the study procedures. Detailed demographics of the final cohort are depicted in Table 1. The majority of twins were women (62%), monozygotic (71%), Caucasian (78%), and of non-Hispanic origin (82%). The median age was 29yr (22–47; IQR) and the median body mass index was 24.3 kg/m^2 (21.8–27.8; IQR). Educational levels were a high school degree for 19, some college education for 95, and a college degree for 114 subjects.

Alfentanil plasma concentrations

The average alfentanil plasma concentration was $72 \pm 16 \text{ ng/ml}$ (SD), which is 28% lower than predicted. The size of this prediction error is consistent with previous results and actual plasma concentrations produced sizable drug effects [4]. While plasma concentrations covered a 3.7-fold range (lowest to highest), about 80% of the concentrations were within a 2-fold range. The plasma concentration was positively correlated with the body mass index ($R = 0.53$; $p < 0.001$) and was significantly higher in men than in women ($76.6 \pm 22.7 \text{ ng/ml}$

versus 62.6 ± 18.8 ng/ml (SD); $p < 0.001$). The body mass index was not different between genders. The average within pair difference in plasma concentration was $17 \pm 14\%$ in monozygotic twins and $21 \pm 16\%$ (SD) in dizygotic twins.

Pain sensitivity

All subjects were successfully trained to perform consistently in pain tests. In most subjects (<90%) 2–3 test cycles were necessary to ascertain consistent performance, while up to 5 test cycles were required in the remaining subjects. Repeating test cycles during the training phase did not result in detectable sensitization to heat-induced pain or desensitization to cold pressor pain, since the heat pain threshold did not decrease significantly, and the cold pressor pain threshold and pain tolerance did not increase significantly with repeated testing.

Heat pain sensitivity

Sensitivity to heat pain varied widely across the studied twin population ranging from 38.7–52.0°C (Figure 2). The median heat pain threshold was 48.4°C (47.0–49.1; IQR). The maximum thermode temperature of 52°C was reached in one subject.

Cold pressor pain sensitivity

Sensitivity to cold pressor pain threshold and cold pressor pain tolerance varied widely across the studied twin population ranging from 2–52s and 5–180s, respectively (Figure 2). Three subjects reached the cut-off of 180s for cold pressor pain tolerance at baseline. The median cold pressor pain threshold was 8.1s (5.9–12.4; IQR) and the median cold pressor tolerance was 16.2s (11.2–25.6; IQR). These values are consistent with previous reports from our laboratory but are lower than those reported by others, which likely reflects methodological differences including variable water temperatures and experimental instructions [2; 25; 56].

Analgesia to heat pain

Analgesic effects exerted by alfentanil varied widely among study participants, being absent in some and resulting in an elevation of the heat pain threshold by up to 5.7°C in others (Figure 3). The median increase in heat pain threshold was 1.2°C (0.7–1.9; IQR; $p < 0.001$). The maximum thermode temperature of 52°C was reached in 9 subjects (8 twin pairs).

Subjects reaching the maximum thermode temperature during administration of alfentanil (ceiling effect) had higher baseline pain thresholds compared with subjects not reaching the maximum thermode temperature ($p < 0.001$). Subjects reaching the maximum thermode temperature had comparable elevation of the heat pain threshold during administration of alfentanil compared with subjects not reaching the maximum thermode temperature. There was a significant negative correlation between the heat pain threshold at baseline and the elevation of the heat pain threshold during the administration of alfentanil ($R = -0.45$, $p < 0.001$; censored data excluded from analysis).

Analgesia to cold pressor pain

Analgesic effects to cold pressor pain varied widely among study participants, being absent in some and resulting in more than a ten-fold increase of the cold pressor threshold and cold pressor tolerance in others (Figure 3). The median increase of the cold pressor threshold was 6.2s (2.7–14.5; IQR; $p < 0.001$) and the median increase in cold pressor pain tolerance was 15.0s (6.8–30.5; IQR; $p < 0.001$). Six subjects (5 twin pairs) already reached the cut-off value of 180s when testing for the effects of alfentanil on the cold pressor pain threshold. Consequently, the effects of alfentanil on cold pressor pain tolerance could not be assessed

in these six subjects. An additional eighteen subjects (16 pairs) reached the cut-off value of 180s when testing for the effects of alfentanil on the cold pressor pain tolerance.

Subjects reaching the cut-off value of 180s during administration of alfentanil had a higher baseline pain threshold and tolerance compared with subjects not reaching the cut-off value ($p<0.001$). Subjects reaching cut-off values showed a greater elevation of the pain threshold and tolerance during administration of alfentanil compared with subjects not reaching the cut-off value ($p=0.012$ and <0.001 , respectively). However, no significant correlation was detected between measures of cold pain sensitivity at baseline (threshold or tolerance) and analgesic effects of alfentanil on these measures (censored data excluded from analysis).

Effects during the infusion of saline placebo

Possible placebo effects were evaluated in the 50% of twins who received the infusion of saline placebo before the infusion of alfentanil. Overall, measures of pain sensitivities changed only modestly during the infusion of saline placebo compared to pre-drug assessments. While some of these changes were statistically significant, they were inconsistent in direction suggesting absence of a relevant placebo effect. A placebo effect would likely affect all analgesic effect measures in similar fashion, i.e., cause an elevation of the heat pain and cold pressor pain threshold as well as an elevation of the cold pressor pain tolerance. Minimal placebo effects are often reported in studies examining analgesic interventions in healthy volunteers under neutral laboratory-type conditions minimizing expectations towards possible outcomes [4]. In the absence of a relevant placebo effect analgesic drug effects were inferred in all subjects by subtracting pre-drug measurements from measurements obtained during the infusion of alfentanil.

The median heat pain threshold before drug infusion was 48.4°C (47.1–49.1; IQR) and slightly increased to 48.5°C (47.4–49.3; IQR) during the infusion of saline placebo ($p=0.001$). The median net-increase of the heat pain threshold from pre-drug measurements was 0.2°C (-0.2 – 0.6 ; IQR) during saline placebo and 1.5°C (0.9 – 2.0 ; IQR) during alfentanil exposure.

The median cold pressor pain threshold before drug infusion was 8.3s (5.6–12.5; IQR) and slightly decreased to 8.0s (5.4–12.1; IQR) during the infusion of saline placebo ($p=0.07$). Similarly, the median cold pressor pain tolerance was 17.9s (10.9–25.0; IQR) before drug infusion and slightly decreased to 15.6s (9.0–22.9; IQR) during the infusion of saline placebo ($p<0.001$). The median net-decrease of the cold pressor pain threshold from pre-drug measurements was -0.3s (-1.3 – 0.6 ; IQR) during saline placebo exposure, while the median net-increase was 7.0s (2.7–16.4; IQR) during alfentanil exposure. Similarly, the median net-decrease of the cold pressor pain tolerance was -1.5s (-3.5 – 0.5 ; IQR) during saline placebo exposure, while the median net-increase was 15.7s (7.9–30.9; IQR) during alfentanil exposure.

Order effects

The order of drug administration did not affect estimates of genetic and environmental effects on studied analgesic effect measures as evidenced by negative results of a separate analysis including the order of drug administration as a fixed covariate ($p=0.13$ – 0.99).

Covariates

Several covariates significantly affected pain sensitivity measures and analgesic drug responses. Results are summarized in Table 2. A description of the demographic covariates is provided in Table 1.

Gender—Women were more sensitive to heat and cold pressor pain than men. Analgesic effects of alfentanil on cold pressor pain tolerance were less pronounced in women than in men.

Age—Older age was associated with decreased pain sensitivity to heat and cold pressor pain. Analgesic effects of alfentanil to heat pain were slightly reduced with older age, while analgesic effects to cold pressor pain were moderately increased.

Race—Caucasians showed decreased pain sensitivity to cold pressor pain as compared to all other races. Analgesic effects of alfentanil to cold pressor pain were more pronounced in Caucasians.

Education—Participants with high school education were less sensitive to heat pain than participants with some college education.

Anxiety—The median POMS anxiety score was 5 (2–7; IQR), which is lower than the score previously reported for a general adult population [63]. Clinically relevant scores, i.e., scores greater than 1.5 standard deviations added to the population mean were noted in 3 women. Increasing anxiety scores were associated with a modest increase in cold pressor pain sensitivity.

Depression—The median Beck Depression Inventory (BDI) score was 3 (1–7; IQR). Eight, three, and two participants had scores compatible with mild, moderate, and severe depression. The BDI was not a significant covariate.

Sleep—The median Pittsburgh Sleep Quality Index was 5 (2–7; IQR). Thirty-six percent of participants had an abnormal PQSI > 6 (range 7–14). Sleep was not a significant covariate.

Blood pressure—The median systolic blood pressure was 116 (108–124; IQR) and the median diastolic blood pressure was 63 (56–71; IQR). Only 2 participants had elevated systolic and diastolic values compatible with the diagnosis of arterial hypertension. An additional 8 participants had elevated systolic values compatible with the diagnosis of isolated systolic arterial hypertension. Arterial blood pressure was not a significant covariate.

Pharmacokinetics—Variations in steady-state alfentanil plasma concentrations were positively correlated with increases in cold pressor pain threshold. However, variations in plasma concentrations were not correlated with increases in heat pain threshold or cold pressor pain tolerance.

Heritability, common environmental and familial effects

Strong heritability was detected for cold pressor pain tolerance and analgesic effects to cold pressor pain threshold. All studied phenotypes showed significant familial effects (genetic and/or shared environmental effects). Estimates of A and C suggest that common environmental effects accounted for a relatively small fraction of observed familial effect. Results are summarized in Table 3.

Pain sensitivity—Significant heritability was detected for cold pressor pain tolerance. Genetic effects accounted for 49% of observed response variance. Significant familial effects were detected for heat and cold pressor pain thresholds. Familial effects accounted for 24 and 32% of observed response variance.

Analgesic effects—Significant heritability was detected for analgesic opioid effects to heat and cold pressor pain. Genetic effects for the drug-induced elevation in heat pain threshold accounted for a modest 12% of observed response variance, while genetic effects for the drug-induced elevation in cold pressor pain threshold accounted for an impressive 60% of observed response variance. Significant familial aggregation was detected for the drug-induced elevation of the cold pressor pain tolerance. Familial effects accounted for 30% of observed response variance.

Heritability, common environmental and familial effects in alternative analyses

Estimates were determined in a secondary analysis using a reduced set of fixed *a priori* covariates including age and gender, and for drug-related outcomes also drug plasma concentration and order of drug infusion. Results were comparable to those obtained when using covariates using the forward selection algorithm described in the method section (Table 4). Importantly, *a priori* inclusion of drug plasma concentration had little impact on results. Neither plasma concentration, nor order of drug infusion passed significance in these analyses.

Estimates were determined in the largest ethnic and racial homogenous subgroup to determine the potential impact of ethnic and racial heterogeneity in our study population. Results were comparable to those obtained in the entire study population (Table 4). While this does not preclude differential genetic contributions to studied phenotypes in distinct ethnic and racial groups, it implies that ethnic and racial heterogeneity in our study population did not significantly distort reported results.

Safety parameters

Systolic arterial blood pressure (BP_{syst}), diastolic arterial blood pressure (BP_{diast}), heart rate (HR), and hemoglobin oxygen saturation (HbSpO₂) remained stable during the infusion of alfentanil as compared to pre-drug measurements. Respective values were 120±18 *versus* 116±13mmHg for BP_{syst}, 63±11 *versus* 64±10mmHg for BP_{diast}, 61±12min⁻¹ *versus* 61±11min⁻¹ for HR, and 99±2 *versus* 98±2% for HbSpO₂. Respiratory rate decreased from 15±3 to 11±3min⁻¹ during the infusion of alfentanil (p<0.001). There were no episodes of arterial hypotension (symptomatic or decrease of mean arterial pressure >25%) or hypoxia (HbSO₂<90%) that required a medical intervention.

Discussion

Large variations in pain sensitivity and analgesic response profiles exist among humans. The increased focus on adequate pain control in the setting of escalating complication rates related to the use of opioids highlight the importance of understanding the reasons underlying such variations. A better understanding will help predict difficulties with pain and pain control in individual patients and allow for the safer and more effective use of opioids. This study used a twin cohort to provide an estimate of the overall contribution of genetic and environmental factors to inter-individual differences in pain sensitivity and analgesic opioid effects. With respect to the analgesic effects of opioids such estimates have not previously been available. The main findings of this study were that significant genetic contributions explained differences in pain sensitivity and analgesic responses. Importantly, the magnitude of the genetic contribution critically depended on the tested pain modality.

The twin study paradigm has occasionally been used for the study of pain. Variably-sized heritable components for disease-associated pain have been reported, ranging from 30–70% in patients suffering from migraine headache, menstrual, pelvic, neck, or back pain [7; 27; 49; 53; 83; 90]. However, results of these studies are difficult to interpret because observed

genetic effects could be due to disease processes rather than pain mechanisms. A few human studies have circumvented this problem by directly examining the genetic contribution to pain sensitivity with the aid of experimental pain models. While experimental pain models do not completely recapitulate clinical pain, their clinical relevance is underlined by studies prospectively linking sensitivity to heat and pressure pain to the magnitude of postoperative pain or the susceptibility to chronic pain disorders [19; 38; 45; 50; 67; 87]. Similarly, carriers of different haplotypic variants of GCH1 and COMT showed concordant variations in experimental and clinical pain sensitivity [23; 81]. Results of our study are generally consistent with previous reports. Nielsen et al. attributed 54% of the variance in cold pressor pain sensitivity to genetic factors, while our study yielded an attributable variance of 49% [59]. A recent family-based study also suggested relevant genetic effects in cold pressor pain sensitivity [9]. Nielsen also reported that 25% of the variance to heat pain sensitivity was attributable to genetic factors. While we found no statistically significant genetic component, 24% of the variance was attributable to significant familial effects (genetic and/or shared environmental effects). Contrasting with Nielsen's study we only measured the heat pain threshold and cannot exclude the possibility that heritability may differ for heat pain exceeding the threshold. A study by Norbury et al. attributed 53% of observed variance in heat pain sensitivity to genetic effects [62]. Only women were enrolled in that study, which may partially account for the discrepant results. Significant gender differences exist for heat pain sensitivity as shown in our study and by others [29]. Available results suggest that substantial genetic components underlay observed differences. However, the particular genetics are likely dependent on the studied pain modality [59]. This highlights the importance of carefully characterizing the particular pain phenotype under study. Cold pressor pain is a particularly attractive modality considering that a large genetic contribution has consistently been demonstrated. Additionally, some studies suggest that variations in cold pressor pain sensitivity and the severity of certain clinical pain are concordant [42; 81].

Differences in cold pressor pain threshold were substantially inherited and the possible genetic underpinnings of this result merit some consideration. One study indicated greater cold pressor pain sensitivity in female carriers of the TRPV1 Ile585Val allele [44]. A subsequent study failed replicating this finding, but identified significant associations between cold pressor pain sensitivity and variants of TRPA1 and COMT in women, and variants of FAAH in men [43]. A study examining the dopamine signaling system reported a significant association between a variant of DRD4 and cold pressor pain sensitivity with a significant interaction between this variant and chronic opioid exposure [37]. A second study failed identifying an association for DRD4, but reported significant associations with Variable Number of Tandem Repeat (VNTR) polymorphisms in the MAO-A and DAT-1 genes [82]. These studies are consistent with our conclusion that genetic factors contribute to the variability in cold pressor pain sensitivity. The available data suggest that a significant number of gene variants determine cold pressor pain sensitivity, each likely accounting for a relatively small fraction of the total variance [24].

The twin paradigm has not previously been applied to the prospective and quantitative study of analgesic opioid responses. Barriers included the relatively large number of required twin pairs and the demands of a pharmacological protocol involving a drug with a significant side effect profile. Nevertheless, the conduct of such studies is feasible and methods necessary for implementing particular experimental protocols have recently been published [3; 78]. This may encourage the more frequent use of the twin study paradigm for pharmacogenomic drug studies. Demonstrating strong heritability for specific phenotypes provides a solid rationale for larger gene association studies.

Few studies have examined the genetics of opioid analgesic response profiles in experimental paradigms. One study examined whether variants of ABCB1 encoding for

drug transporter p-glycoprotein could alter cold pressor pain responses, supposedly by changing drug distribution in the central nervous system, and reported more pronounced analgesic effects of oxycodone in carriers of the G2677T variant [91]. Our most prominent result suggests that 60% of the variance observed for opioid-mediated elevation of the cold pressor threshold were attributable to genetic effects. We detected no statistically significant heritable component for opioid-mediated elevation of the cold pressor tolerance. However, 30% of the observed variance was attributable to familial effects, which include possible genetic effects. The analysis of opioid-mediated analgesic effects on cold pressor pain tolerance was also impacted by ceiling effects in 24 subjects, which may have affected estimates of variance and heritability.

Several other studies have explored the influence of gene variants on opioid analgesia in the setting of clinical pain or in experimental settings using modalities other than cold pressor pain. Many of the relevant findings have been summarized in recent reviews [40; 64; 77; 86]. Best studied is the A118G polymorphism located on OPRM1. Studies in patients undergoing surgery, though generally small-sized, have fairly consistently identified increased opioid requirements in carriers of the G allele [16; 17; 36; 76]. Of particular interest is a study documenting increased requirements of alfentanil, the drug used in our study, in carriers of the G allele when undergoing lithotripsy [33]. Despite the available reports, the clinical relevance of associations between the A118G variant and opioid requirements remains unclear [86]. As importantly, only a small number of gene variants have been explored in the context of opioid analgesia, making it likely that relevant variants have not yet been detected. Finally, results of studies examining gene variants are not generally consistent, which may partially be explained by the small effect size attributable to a single variant, limited statistical power, ethnic admixture and heterogeneity of studied phenotypes. Available results suggest that substantial genetic components underlay observed differences in analgesic opioid effects. The particular genetics likely involve multiple variants and depend on the type of studied pain. Cold pressor pain is a particularly attractive to study the genetics of opioid analgesia considering that it is a homogenous phenotype with a strong heritable component.

While our study was not adequately powered to examine genetic effects in subgroups including gender and ethnicity (other than non-Hispanic Caucasians), it allowed examining the contributions of several covariates to studied phenotypes. Key findings confirm previous results emphasizing the importance of accounting for effects of age, gender, anxiety and race in studies of pain and analgesia. Consistent with earlier reports heat and cold pressor pain thresholds increased with advancing age [32]. Similarly, increased sensitivity to threshold or supra-threshold noxious stimuli was associated with female gender, anxiety, and race other than Caucasian [14; 26; 29; 85]. Generally, studies examining the effects of gender report similar or somewhat higher analgesic potencies in women [60]. Caucasians displayed increased analgesic opioid effects to cold pressor pain. This is an unexpected finding considering that Caucasians undergoing surgery require more opioids [21; 58]. The finding may also be influenced by the composition of the non-Caucasian group, which was mainly Asian in our study. Finally, depression and arterial blood pressure were not significant covariates in our study despite previous reports linking these variables to pain sensitivity and severity [12; 74]. Our negative findings may be explained by the fact that few participants had clinically relevant elevations in depression scores or blood pressure.

Substantial interest in identifying factors linked to the large variability in pain sensitivity and analgesic opioid responses exists. Our study demonstrates that genetic and environmental factors as well as key covariates alter pain sensitivity and analgesic opioid responses, but that their relative contributions depend on the pain modality under study. Based on our results, the cold pressor pain model may be particularly amenable to genetic analysis. Our

data support larger-scale molecular genetic studies in humans to discover key elements contributing to pain and opioid analgesic response profiles.

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Figure 1.

Two hundred and twenty eight monozygotic and dizygotic twins successfully underwent a computer-controlled infusion with the mu-opioid agonist alfentanil in a single occasion, randomized, double-blinded and placebo-controlled study paradigm. Baseline assessments included covariates affecting pain perception, sensitivity to experimental heat and cold pressor pain, vital signs, and blood draws. Fifty percent of twin pairs were allocated to receive alfentanil first and saline placebo second, while the other fifty percent of twin pairs received alfentanil and saline placebo in reversed order. The alfentanil target concentrations for both treatment sequences are depicted in the graph. A concentration of 100ng/ml produces significant analgesic effects in patients suffering from postoperative pain. Analgesic drug effects and vital signs were assessed in identical fashion during both stages of the infusion protocol.

Figure 2.

Pain sensitivity was assessed with aid of a heat and cold pressor pain model. Heat pain sensitivity was measured by determining the minimum thermode temperature evoking pain while in contact with skin (threshold). Cold pressor pain sensitivity was determined by measuring the time to the onset of pain (threshold) and the time to intolerable pain (tolerance), while the hand and parts of the forearm were immersed in ice water (re-circulated and kept at 1–2°C). Results are ranked from smallest to largest along the x-axis. The inter-individual differences in heat pain sensitivity varied widely and covered a range greater than 10°C. Similarly, inter-individual differences in cold pressor pain sensitivity varied widely and covered more than a ten-fold range. The solid and dashed lines indicate the median and the interquartile range, respectively.

Figure 3.

Administration of alfentanil resulted in significant analgesic effects to heat and cold pressor pain ($p < 0.001$). Results are ranked from smallest to largest along the x-axis. Analgesic effects varied widely among study subjects. Analgesic effects to heat pain were absent in some subjects but resulted in a maximum elevation the heat pain threshold of up to 5.7°C in others. Similarly, analgesic effects to cold pressor pain were absent in some subjects but resulted in an elevation of the cold pressor pain threshold and cold pressor pain tolerance to the cut-off value of 180s. The solid and dashed lines indicate the median and the interquartile range, respectively.

Table 1

Demographic characteristics

Category	Subcategories	N (pairs)	
		MZ	DZ
Zygosity	Total	81	33
Gender	Women	52	12
	Men	29	8
	Mixed	-	13
Age	18–30	40	21
	31–40	13	3
	41–50	9	3
	51–60	13	2
	61–70	6	4
Race	African American	3	2
	American Indian	1	1
	Asian	12	5
	Caucasian	64	25
	Pacific Islander	1	0
Ethnicity	Hispanic	14	6
	Non-Hispanic	67	27

DZ = dizygotic, MZ = monozygotic

Table 2

Covariates

Phenotype ¹	Covariate	Effect ²	p-value
Pain sensitivity			
Heat pain threshold (°C)	Age	+ 0.44°C per decade	<0.001
	Gender	+ 0.53°C men <i>versus</i> women	0.032
Cold pressor pain threshold (s)	Age	+ 6.5% per decade	0.014
	Race	+ 22.3% Caucasian <i>versus</i> others	0.040
	Mood	− 0.8% per unit (POMS)	<0.001
Cold pressor pain tolerance (°C)	Gender	+ 23.3% men <i>versus</i> women	0.015
	Race	+ 27.3% Caucasian <i>versus</i> others	0.028
	Mood	− 0.8% per unit (POMS)	0.004
Analgesia			
Increase in heat pain threshold (°C)	Age	− 0.13°C per decade	0.008
	Education	+ 0.48°C high school only <i>versus</i> college	0.024
Increase in cold pressor pain threshold (s)	Alfentanil	+ 0.5% per ng/ml of plasma	0.014
	Race	+ 19.8% Caucasian <i>versus</i> others	0.010
Increase in cold pressor pain tolerance (s)	Age	+ 7.4% per decade	0.018
	Gender	+ 24.8% men <i>versus</i> women	0.012

¹°C = degree Celsius, s = seconds,

²Race: others include African American, American Indian, Asian, Pacific Islander; POMS: profile of mood states subscale for anxiety; (range 0–36)

Table 3

Genetic (A), common environmental (C), and familial (A+C) effects

Phenotype ¹	Hypothesis tests ²		Estimates under ACE model ³		Covariates ⁴
	Heritability	Familial Aggregation	A	C	
Pain sensitivity					
Heat pain threshold (Cel)	0.305	0.010	20 (0–38)	4 (0–35)	24 (6–41) A, G
Cold pressor pain threshold (s)	0.310	<0.001	17 (0–47)	15 (0–36)	32 (11–50) A, M, R
Cold pressor pain tolerance (s)	0.007	<0.001	49 (14–61)	0 (0–27)	49 (30–61) G, M, R
Analgesia					
Increase in heat pain threshold (Cel)	0.005	0.446	12 (0–32)	0 (0–0)	12 (0–32) A, E
Increase in cold pressor pain threshold (s)	0.004	<0.001	60 (23–73)	0 (0–27)	60 (35–74) R
Increase in cold pressor pain tolerance (s)	0.162	0.002	30 (0–47)	0 (0–35)	30 (10–48) A, G

¹Cel = degree Celsius, s = seconds

²P-values

³Percentage and 95%-confidence interval

⁴A = age, E = education, G = gender, M = mood (anxiety), R = race; p<0.05

Table 4
Genetic (A), common environmental (C), and familial (A+C) effects in alternative analyses

Phenotype ¹	Hypothesis tests ²		Estimates under ACE model ³		
	Heritability	Familial Aggregation	A	C	A+C
Heat pain threshold (Cel)					
All subjects, selected covariates	0.305	0.010	20 (0–38)	4 (0–35)	24 (6–41)
All subjects, fixed covariates	0.305	0.010	20 (0–38)	4 (0–35)	24 (6–41)
Caucasians ⁴ only, selected covariates	0.134	0.004	39 (0–54)	0 (0–48)	39 (17–56)
Cold pressor pain threshold (s)					
All subjects, selected covariates	0.310	<0.001	17 (0–47)	15 (0–36)	32 (11–50)
All subjects, fixed covariates	0.169	<0.001	31 (0–51)	5 (0–36)	36 (15–52)
Caucasian ⁴ only, selected covariates	0.367	<0.001	13 (0–51)	21 (0–41)	34 (13–54)
Cold pressor pain tolerance (s)					
All subjects, selected covariates	0.007	<0.001	49 (14–61)	0 (0–27)	49 (30–61)
All subjects, fixed covariates	0.005	<0.001	53 (17–64)	0 (0–29)	53 (35–65)
Caucasian ⁴ only, selected covariates	0.001	<0.001	57 (26–69)	0 (0–26)	57 (38–69)
Increase in heat pain threshold (Cel)					
All subjects, selected covariates	0.005	0.446	12 (0–32)	0 (0–0)	12 (0–32)
All subjects, fixed covariates	0.008	0.603	7 (0–26)	0 (0–0)	7 (0–26)
Caucasian ⁴ only, selected covariates	0.019	0.558	9 (0–33)	0 (0–18)	9 (0–34)
Increase in cold pressor pain threshold (s)					
All subjects, selected covariates	0.004	<0.001	60 (23–73)	0 (0–27)	60 (35–74)
All subjects, fixed covariates	0.005	<0.001	60 (17–73)	0 (0–28)	60 (31–73)
Caucasian ⁴ only, selected covariates	0.023	<0.001	65 (0–82)	0 (0–47)	65 (33–83)
Increase in cold pressor pain tolerance (s)					
All subjects, selected covariates	0.162	0.002	30 (0–47)	0 (0–35)	30 (10–48)
All subjects, fixed covariates	0.174	0.004	30 (0–46)	0 (0–36)	30 (7–48)
Caucasian ⁴ only, selected covariates	0.361	<0.001	19 (0–56)	20 (0–49)	38 (14–49)

¹Cel = degree Celsius, s = seconds

²P-values

³Percentage and 95%-confidence intervals

⁴Non-Hispanics only