Epigenetic Regulation of Chronic Pain
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Multicenter study of *OPRM1* A118G and promoter-region DNA methylation associations with opioid outcomes and chronic postsurgical pain after pediatric musculoskeletal surgery

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Abstract

Introduction: Mu opioid receptor gene (*OPRM1*) variant rs1799971 introduces a CpG site, which may influence DNA methylation (DNAm) and opioid/pain outcomes.

Objectives: In this nested analysis, we investigated both *OPRM1* A118G genotype and promoter/immediate downstream blood DNAm sequencing data for associations with opioid effects and chronic postsurgical pain (CPSP) in a surgical cohort.

Methods: Prospectively recruited opioid naïve patients undergoing Nuss procedure or spinal fusion with rs1799971 genotypes (Illumina arrays), DNAm (next generation enzymatic methylation sequencing at Chr6:154,039,209-154,039,803) and outcomes—opioid analgesia (integrated opioid use + pain over postoperative days 0 and 1 normalized to surgery type), safety—respiratory depression (RD) in high opioid use groups, and CPSP (Numerical Rating Scale >3/10 2-12 months postsurgery)—were included. Linear and logistic regression were performed to test genetic and epigenetic associations, adjusted for sociodemographics, cell types, and analgesics.

Results: In this cohort (N = 112; 15.3 ± 2.0 years, 50% female, 83% White, 55% had CPSP, 13% had RD), DNAm at Chr6: 154039216-154039217 was associated with CPSP (odds ratio [OR], 1.26; 95% confidence interval [CI], 1.00-1.57; $P = 0.03$), Chr6: 154039661-154039662 with acute integrated pain ($\beta = -20.9$, 95% CI, −40.70 to −1.10, $P = 0.04$), Chr6:154039520-154039521 (OR, 1.49; 95% CI, 1.09-2.03; $P = 0.01$), and Chr6:154039571-154039572 (OR, 1.47; 95% CI, 1.08-2.01; $P = 0.02$) with RD. Significant CpG sites were located in Repressed Polycomb chromatin states. Genotype was not associated with DNAm or outcomes.

Conclusion: Our analyses support *OPRM1* DNAm as predictors of acute and chronic pain/opioid outcomes in children after painful surgery. Study limitations included absent GG genotype, low sequencing coverage, and lack of correction for multiple testing.

Keywords: *OPRM1*, Genetics, Epigenetics, Opioid effects, Chronic postsurgical pain, DNA methylation

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

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1. Introduction

In the United States, ≈3.9 million pediatric surgeries are performed annually.[42] Despite advances in pain management, postsurgical pain control remains inadequate[4,63] and is often associated with chronic postsurgical pain (CPSP), defined as pain lasting beyond 2 to 3 months after surgery.[62] Reported incidence of CPSP ranges from ~11% to 60%,[15,17,20,45] with negative economic,[21] health, and social-emotional consequences.[36,43,45] Although CPSP was recently included in the *International Classification of Diseases, 11th Revision*,[49,55] pediatric CPSP is less well studied.[19] While multimodal therapy to minimize opioid side effects is common practice, opioid analgesics remain the mainstay for treating severe surgical pain. However, high interindividual variability in opioid responses can affect both analgesic efficacy[54] and adverse outcomes, such as respiratory depression (RD),[3] thus affecting provision of safe analgesia.[41]

Both genetic and epigenetic variants have been implicated in interindividual variability of pediatric pain and opioid responses.[13,14,16,17] Epigenetic modifications, such as DNA methylation (DNAm), can regulate gene expression.[22] The mu opioid receptor gene (*OPRM1* [MIM: 600018]) is an important gene involved in opioid signaling. It encodes the mu opioid receptor (MOR), the primary target of endogenous and exogenous opioids.[68] Mu opioid receptor is expressed in brain, peripheral pain pathways,[37,68] and in blood immune cells.[7,10,26,64] Prior studies have shown that *OPRM1* expression is decreased by DNAm of its promoter region[29] and by the presence of the missense single nucleotide polymorphism (SNP) (p.Asn40Asp), commonly known as A118G or rs1799971.[39] However, association studies between variant rs1799971 and opioid consumption, pain responses,[23,30,33,60,67] and risk of RD[13] have yielded inconsistent results.[13,18,23,69] Furthermore, the A to G mutation of rs1799971 has been reported to introduce a CpG methylation site (Chr6:154,389,662) and enhance methylation at this site and downstream.[39] We previously reported associations of rs1799971 A>G with increased opioid requirement and decreased effects of morphine[13] and associations of DNAm at *OPRM1* promoter CpG sites (by pyrosequencing) with acute and CPSP after posterior spine fusion (PSF) in pediatric patients at a single site.[16] However, no single study has investigated that both variant rs1799971 and *OPRM1* promoter region DNAm influences on pediatric postsurgical pain and opioid responses.

We conducted a prospective, observational, multisite study in adolescents undergoing spine and pectus deformity surgeries. We hypothesized that G allele carriers of A118G and DNAm in *OPRM1* promoter and immediate downstream regions would be associated with lower opioid responses, namely, higher acute integrated pain profiles (opioid consumption and pain), lower risk for RD, and higher risk for CPSP. We also aimed to determine if genotype is associated with DNAm at the CpG site introduced by rs1799971 A>G and explore genetic–epigenetic interactions on opioid outcomes.

2. Methods

2.1. Approval

This observational, prospective study was approved by Cincinnati Children's Hospital as the single institutional review board and then approved by respective institutional review boards at the other 5 sites. This study was registered at ClinicalTrials.gov NCT01839461, NCT01731873. Informed consent was obtained from parents or guardians and assent was obtained from subjects before enrollment in each study.

2.2. Inclusion and exclusion criteria

Subjects aged 8 to 21 years with a diagnosis of adolescent idiopathic scoliosis or pectus excavatum scheduled to undergo posterior spinal fusion or endoscopic pectus excavatum repair respectively were included. They were prospectively recruited between 2016 and 2021. Subjects were excluded from participating if they (1) had renal or liver disease, (2) used opioids in the last 2 years, (3) had severe respiratory problems or cardiac conditions, (4) were pregnant or breastfeeding, (5) were non-English speaking, or (6) had severe developmental delays.

2.3. Data collection

2.3.1. Demographics

Patient characteristics (age, sex, race, weight, residential address, family highest education, and annual income) were collected preoperatively. Neighborhood deprivation index (DI) was calculated based on ZIP code tabulation area level data.[9] Deprivation index explains ~70% of variance in census tract level socioeconomic variables (poverty, education, health insurance coverage, public assistance and vacant homes, median household income) from the American Community Survey.

2.3.2. Surgery and analgesia data

Details with respect to surgery (surgical duration) and pain management (in-hospital opioid consumption, patient-controlled analgesia, intravenous and oral opioids administered over postoperative days [POD] 0 and 1), use of analgesic adjuvants (acetaminophen, nonsteroidal anti-inflammatory drugs, gabapentin/pregabalin, regional anesthesia, methadone, ketamine, and dexmedetomidine) were obtained from medical records. Opioid consumption over POD 0 and 1 was quantified as morphine equivalents (MEq/kg) adjusted for weight.

2.3.3. Pain assessments

Nurses trained in the use of the Numerical Rating Scale (0-10)[58] documented pain scores at least every 4 hours postoperatively. These pain scores were obtained from the electronic medical record. Acute postoperative pain was measured as the area under the curve (AUC) of Numerical Rating Scale pain scores over POD 0 and 1. PainDETECT, a questionnaire designed to detect neuropathic pain,[24] was administered preoperatively, 2 to 6 and 10 to 12 months after surgery. Patients reported current, average, and maximum pain over last 4 weeks and location of pain on the questionnaire.

2.3.4. Psychological risk assessment

The Pediatric Pain Screening Tool (PPST) was administered preoperatively to assess psychological risk for CPSP. Pediatric Pain Screening Tool is a simple 9-item questionnaire with physical and psychosocial scale subscores. It has been previously validated for rapid identification of risk for poor pain outcomes in youth with pain,[53] and we previously found that a PPST score ≥2 was associated with high risk (48.94%) of CPSP in a pediatric cohort undergoing PSF or Nuss procedure.[38]

2.3.5. Respiratory assessments

Patients were monitored while in-hospital using pulse oximetry and transthoracic impedance plethysmography. Oxygen saturation and respiratory rate data were captured in the electronic medical record and subsequently reviewed. In addition, use of oxygen supplementation (per notes and chart) was also captured.

2.3.6. Outcomes

Chronic postsurgical pain was the primary outcome. Question 2 of PainDETECT "How strong was the strongest pain during the past 4 weeks?" was used to determine CPSP (positive if pain ≥3/10 at 2- to 6- or 10- to 12-month questionnaires).

Acute integrated pain outcome and RD were secondary opioid efficacy and safety outcomes. Opioid doses and acute pain scores together give a comprehensive picture of pain, which can differ by surgery. As both metrics can potentially be affected by *OPRM1* genomics, we used the Silverman approach[52] to generate integrated outcomes for opioid analgesic efficacy to enable comparison of overall normalized pain generalizable to both surgical cohorts. For this, AUC and MEq/kg POD 0 and 1 were ranked by surgery type and ranks were summed to get an integrated outcome independent of surgery type. Respiratory depression was defined as having a respiratory rate ≤8 to 10 per minute, $SpO_2 < 90\%$ or need for oxygen beyond 4 hours over POD 0 and 1.

2.4. Genotyping and DNA methylation

Blood samples were collected preoperatively and sent to Cincinnati Children's Hospital Medical Center (CCHMC) Molecular Genetics Laboratory (Cincinnati, OH) for DNA isolation. CCHMC DNA Sequencing and Genotyping Core (Cincinnati, OH) performed genotyping on the Illumina Omni5-4 v1.0 and next generation enzymatic methylation sequencing (NG EM-seq) on the Illumina NovaSeq. Next generation enzymatic methylation sequencing reads were aligned to the GRCh38/hg38 (GCF_000001405.26) genome or a version of the genome containing the SNP rs1799971 A>G using bismark 0.22.3. We identified methylation at CpG sites (5′ position of a cytosine residue followed by a guanine residue) and non-CpG sites (in which a methylated cytosine can be followed by an A, T, or C, known as CHH or CHG [where H = A, T, or C]). Methylation calls (Methylkit 1.20.0, coverage ≥4) of sense and antisense adjacent CpG cytosines were averaged together (see Supplementary Methods, available at http://links.lww.com/PR9/A255 for more details).

2.5. Cell-type deconvolution

Blood cell counts were obtained by cell-type deconvolution, which was performed from the NG EM-seq methylation calls, using EpiDish 2.14.1 with FlowSorted.BloodExtended.EPIC created by Salas et al.[47] as the reference library. This produced estimated cell-type fractions of 12 blood immune cell types per patient for use as covariates.

2.6. Statistical and functional analyses

2.6.1. Genotype and DNA methylation association with outcomes

All analyses were conducted using SAS 9.4. Before analysis, the quality and distribution of data were examined. Univariable analysis was then performed to test the association between each outcome and each potential influencing factor (age, sex, race, preoperative PPST, preoperative pain, use of each multimodal analgesic, DI, family education and income, surgical duration, and cell counts (for DNAm) were considered as covariates). Binary bins for race (Whites vs non-Whites), education (more than high school vs less), and income (less than $29,999 per year vs more) were used. Factors associated at $P = 0.1$ level were included in subsequent multivariable analysis. Random effect of study site was tested using linear or generalized linear mixed model; no statistically significant effect was detected for CPSP or RD. Therefore, logistic regression was used to test the genetic and epigenetic effects on CPSP and RD, and linear mixed model on acute integrated pain score. Associations of significant covariables with genotype and DNAm were checked for collinearity. Association of genotype and methylation% on outcomes were tested adjusting for significant covariables, with or without including the cell types. Statistical significance was considered at $P < 0.05$ level. Results should be interpreted with caution for inflated type I error.

2.6.2. Functional analyses

CpG sites with statistically significant associations with outcomes were visualized in WashU Epigenome Browser.[34,35] The "JASPAR Transcription Factors 2022" track was loaded to identify transcription factors (TFs) predicted to bind to these sites[44] selected by position weight matrices relative score ≥0.8 and a *P*-value <0.05. Reference human epigenomes from Roadmap Epigenomics Consortium "all chromatin states" tracks of blood cells were also loaded into WashU Epigenome Browser to visualize chromatin states. Molecular interactions between these TFs and *OPRM1* were elucidated in QIAGEN IPA (QIAGEN Inc, https://digitalinsights.qiagen.com/IPA) using Path builder function and pathways in which the molecules are involved were identified.

3. Results

3.1. Patient demographics and characteristics

Of 322 subjects recruited between 2016 and 2021, retention was 76.5%. Of those who had outcomes, 180 had genotypes, and 112 had both genotype and DNAm data (included in this analysis). We compared the 112 subjects with those excluded in the overall cohort and the cohort with outcomes for age, AUC (analysis of variance); surgical duration, PPST, opioid use, and preoperative pain (Kruskal–Wallis); sex, race, genotype, surgery type, and CPSP (χ^2 test). Excluded cohorts had a higher proportion of PSF subjects (89% vs 53%, $P < 0.001$). Further comparison stratified by surgery type showed no differences in pectus cohort, while in the PSF group, the excluded cohort was younger ($P = 0.01$) but did not differ by other variables. Most (58.9%) participants were recruited from 1 site (CCHMC). Demographics and variable characteristics for the cohort are given in **Table 1**. Subjects ranged in age from 9 to 21 years of age (50% female, 83% Whites). In this cohort, the incidence of CPSP was 55% and RD occurred in 13%. Pain scores reported at 2 to 6 and 10 to 12 months were highly correlated ($R^2 = 0.44$, $P < 0.001$), showing consistency in pain experience over time. In addition, the body part where pain was reported on the questionnaire was confirmed to be surgery related. At *OPRM1* SNP rs1799971, 28% were heterozygous (AG), while 72% were wild type (AA). Medication use is described in **Table 2**.

Table 1

Patient sociodemographics and characteristics.

Variables	Overall (n = 112)
Age (y), mean (SD)	15.3 (2.0)
Sex, n (%)	
Female	56 (50)
Race, n (%)	
Asian	5 (5)
Black	10 (9)
Native Hawaiian or other Pacific Islander	1 (1)
Other	1 (1)
Unknown	2 (2)
Caucasian	92 (83)
Weight (kg), mean (SD)	56.0 (11.1)
Deprivation index, mean (SD)	0.3 (0.1)
Education, n (%)	
Less than high school	3 (4)
High school	10 (14)
Some college	11 (16)
Bachelor's degree	18 (26)
Graduate degree	23 (33)
I prefer not to provide	4 (6)
Annual income, n (%)	
Less than $14,999	0
$15,000-29,999	5 (7)
$30,000-44,999	1 (1)
$45,000-59,999	4 (6)
$60,000-89,999	3 (4)
$90,000-119,000	11 (16)
More than $120,000	30 (45)
I prefer not to provide	13 (19)
Genotype and epigenetics based cell counts	
rs1799971, n (%)	
AA	81 (72)
AG	31 (28)
Cell counts, median (IQR)	
Basophils	0.001 (0.000-0.016)
B memory	0.013 (0.004-0.021)
B naïve	0.046 (0.028-0.068)
CD4 memory	0.049 (0.026-0.083)
CD4 naïve	0.090 (0.054-0.129)
CD8 memory	0.018 (0.000-0.041)
CD8 naïve	0.042 (0.016-0.074)
Eosinophils	0.000 (0.000-0.000)
Monocytes	0.066 (0.043-0.096)
Neutrophils	0.553 (0.453-0.638)
Natural killer	0.042 (0.027-0.062)
T regulatory	0.015 (0.001-0.034)

IQR, interquartile range.

Table 2

Pain and perioperative characteristics.

Preoperative pain and psychosocial measure	
Pediatric pain screening tool score, median (IQR) [b]	1 (0-3)
Preoperative pain (NRS >0), n (%)	
No	81 (75)
Yes	27 (25)
Surgery characteristics	
Surgery, n (%)	
Pectus	52 (46)
Spine	60 (54)
Site of recruitment, n (%)	
1	66 (59)
2	24 (21)
3	7 (6)
4	4 (4)
5	7 (6)
6	4 (4)
Surgical duration (min), median (IQR)	178.0 (91.0-242.5)
Perioperative medication use	
Use of acetaminophen, n (%) [c]	
Yes	101 (90)
No	11 (10)
Use of ketorolac, n (%)	
Yes	97 (87)
No	15 (13)
Use of remifentanil, n (%)	
Yes	33 (31)
No	75 (69)
Use of gabapentinoids, n (%)	
Yes	89 (79)
No	23 (21)
Use of ketamine, n (%)	
Yes	95 (85)
No	17 (15)
Use of dexmedetomidine, n (%)	
Yes	90 (80)
No	22 (20)
Pain and opioid outcomes	
Opioid consumption over postoperative days 0-1, median (IQR)	0.9 (0.7-1.4)
Acute pain area under the curve over postoperative days 0-1, mean (SD)	184.2 (80.4)
Acute integrated pain, mean (SD)	−5.5 (85.2)
Respiratory depression, n (%)	
Yes	14 (13)
No	98 (88)
Chronic postsurgical pain, n (%)	
Yes	50 (45)
No	62 (55)

IQR, interquartile range; NRS, Numerical Rating Scale.

3.2. OPRM1 *CpG sites selection*

Inspection of the average CHH (H = A, C, or T), CHG, and CpG methylation across the genomic region spanning the vicinity of the first 4 exons of *OPRM1* from Chr6:154,000,000-154,130,000 (**Fig. 1**, top panel) revealed dense CpG hypomethylation at exon 1, but not at other potential transcription start sites (TSSs) that encode isoforms of *OPRM1*. Within the *OPRM1* promoter region, average (±SEM) cytosine coverage was 7.84% ± 1.3%. CpG-specific methylation at Chr6:154,039,209-154,039,803, spanning 41 symmetrical CpG sites in *OPRM1* promoter and immediate downstream regions and including the SNP rs1799971, was chosen for all subsequent analyses (**Fig. 1**, bottom panel). For brevity, sites are abbreviated by their final 3 digits (ie, XXX from Chr6: 154,039,XXX) for the remainder of this manuscript.

3.3. OPRM1 *rs1799971: DNA methylation associations*

DNA methylation levels at each CpG site by genotype are presented in **Figure 2**. Further quantification of DNAm values at each CpG site is provided in Supplementary Figure S1 (available at http://links.lww.com/PR9/A255) and a heatmap of correlations between DNAm at all sites in Supplementary Figure S2 (available at http://links.lww.com/PR9/A255). DNA methylation at CpG 520-521 showed weak correlations (R^2 = 0.3-0.4) with DNAm at 4 other sites. Genotype was not associated with race or biological sex; thus, genotype associations with outcomes were tested using Wilcoxon method without adjusting and no associations were found.

3.4. *Chronic postsurgical pain associations*

Univariate analysis showed sex effect on CPSP outcome (Supplementary Table 1, available at http://links.lww.com/PR9/A255), with 61% of those developing CPSP being female (P = 0.001).[39] No association of sex was found with DNAm ($P > 0.05$ for all 41 CpGs, t test) or genotype. rs1799971 was not

Table 3

Significant CpG site methylation associations with outcomes.

		Regression coefficient (SE)	*P*	OR/change (95% CL)
Chronic postsurgical pain[a]				
Chr6:154039216-154039217	Promoter	4.56 (2.31)	0.03	1.26 (1.00 to 1.57)
Acute integrated pain[b]				
Chr6:154039661-154039662	SNP, past TSS in first exon	−417.97 (198.04)	0.04	−20.90 (−40.70 to −1.10)
Respiratory depression[a]				
Chr6:154039520-154039521	Immediately before TSS in first exon	8.00 (3.17)	0.01	1.49 (1.09 to 2.03)
Chr6:154039571-154039572	Immediately past TSS in first exon	7.72 (3.20)	0.02	1.47 (1.08 to 2.01)

Association was tested using [a]logistic regression; [b]linear mixed model. OR change in acute integrated pain was for 5% increase in DNA methylation.
CL, confidence limit; OR, odds ratio; SNP, single nucleotide polymorphism; TSS, transcription start site.

associated with CPSP before or after adjusting for sex ($P = 0.3$). DNA methylation of CpG site 216-217 was associated with CPSP ($P = 0.03$, odds ratio [OR], 1.26 [95% confidence interval (CI), 1.00-1.57] for 5% increase of DNAm) (**Table 3**, **Fig. 3**). The DNAm of CpG site 216-217 association still existed when cell types were included in the modeling. No interaction was detected between CpG site 216-217 and rs1799971 for CPSP.

3.5. Acute integrated pain associations

We identified significant correlations between DNAm and opioid consumption (MEq/kg) at CpG 531-532 (Spearman $r = 0.199$, $P = 0.04$) and 348-349 (Spearman $r = 0.227$, $P = 0.02$). Single nucleotide polymorphism rs1799971 was not associated with MEq/kg ($P = 0.7$). We also found that DNAm at CpG 661-662 was negatively associated with AUC (1% increase in DNAm was associated with −48.1 change in AUC, $P = 0.034$), after adjusting for age, preoperative pain, preoperative PPST, and cell counts. As shown in Supplementary Table 2 (available at http://links.lww.com/PR9/A255), univariate analyses identified factors that may influence acute integrated pain, including preoperative PPST (Pearson $r = 0.298$, $P = 0.003$), acetaminophen administration ($P < 0.002$, *t* test), B-cell naïve (Pearson $r = −0.185$, $P = 0.06$), and dexmedetomidine use ($P = 0.008$, *t* test). None of the above covariates associated with rs1799971 at $P = 0.05$ level. No genetic effect ($P = 0.82$) was detected after adjusting the covariates and accounting for the random site effect. After adjusting for covariates, DNAm association was found for CpG site 661-662 with acute integrated pain ($P = 0.04$, acute integrated pain decreased by 20.9 [95% CI, −40.70 to −1.10] for 0.05% increase in DNAm) (**Table 3**, **Fig. 3**). After adjusting for cell types, epigenetic effect was noted for CpG sites 209-210 ($P = 0.03$, acute integrated pain decreased by 9.8 for 0.05% increase in DNAm), 597-598 ($P = 0.04$, acute integrated pain decreased by 4.9 for 0.05% increase in DNAm), and CpG site 661-662 ($P = 0.09$). No genetic–epigenetic interaction was found.

3.6. Respiratory depression associations

Univariate analyses suggested effects of sex, surgery type, and opioid use on RD (Supplementary Table 3, available at http://links.lww.com/PR9/A255). Respiratory depression rate may also differ by study sites. Due to the correlations among the significant covariates, we tested them in a generalized linear mixed model and determined that MEq/kg was the most important covariate and random site effect was not significant. After adjusting for MEq/kg in a multiple logistic regression, no genetic association was found ($P = 0.9$), but an epigenetic association was detected at CpG site 520-521 ($P = 0.01$; OR, 1.49; [95% CI, 1.09-2.03] with 5% increase in DNAm) and 571-572 ($P = 0.02$; OR, 1.47 [95% CI, 1.08-2.01] with 5% increase in DNAm) (**Table 3**, **Fig. 3**). After adding cell types to the model, the epigenetic association still existed for these 2 CpG sites. No genetic–epigenetic interaction was found.

3.7. Functional relevance

For each of the 4 CpG sites significantly associated with CPSP, acute integrated pain, or RD (**Table 3**), TFs predicted to bind at the site were identified (**Table 4**). For example, the TFs predicted to bind at the 216-217 CpG site associated with CPSP were Gmeb1, CEBPD, CEBPG, and CEBPA. Furthermore, all 4 significant CpG sites were found to be located in areas of the genome marked by chromatin states of RepressedPolycomb or Weak RepressedPolycomb in all peripheral blood cells, except primary B cells and primary T helper memory cells in which this signal was quiescent at the CpG sites.

IPA path builder's function revealed molecules known to interact with both the TFs predicted to bind at each of the 4

Table 4

Predicted transcription factor binding to the CpG positions identified in pain and opioid responses.

Outcomes	CpG site	Transcription factors (JASPAR matrix ID)
Chronic postsurgical pain	Chr6:154039216-154039217	Gmeb1 (MA0615.1), CEBPD (MA0836.2), CEBPE (MA0837.2), CEBPG (MA0838.1), CEBPA (MA0102.4)
Acute integrated pain	Chr6:154039661-154039662	MEIS3 (MA0775.1), NR2C2 (MA1536.1)
Respiratory depression	Chr6:154039520-154039521	OVOL2 (MA1545.1), Tcf12 (MA0521.2), Bhlha15 (MA1472.2), Ptf1A (MA1619.1), MYF5 (MA1641.1), NHLH1 (MA0048.2), Twist2 (MA0633.2)
	Chr6:154039571-154039572	Foxn1 (MA1684.1), MEIS1 (MA0498.2), NFIX (MA0671.1)

Annotation was performed using WashU Epigenome Browser with track JASPAR Transcription Factors. JASPAR matrix IDs are provided and may be used to look up the original data sources used in JASPAR curations.

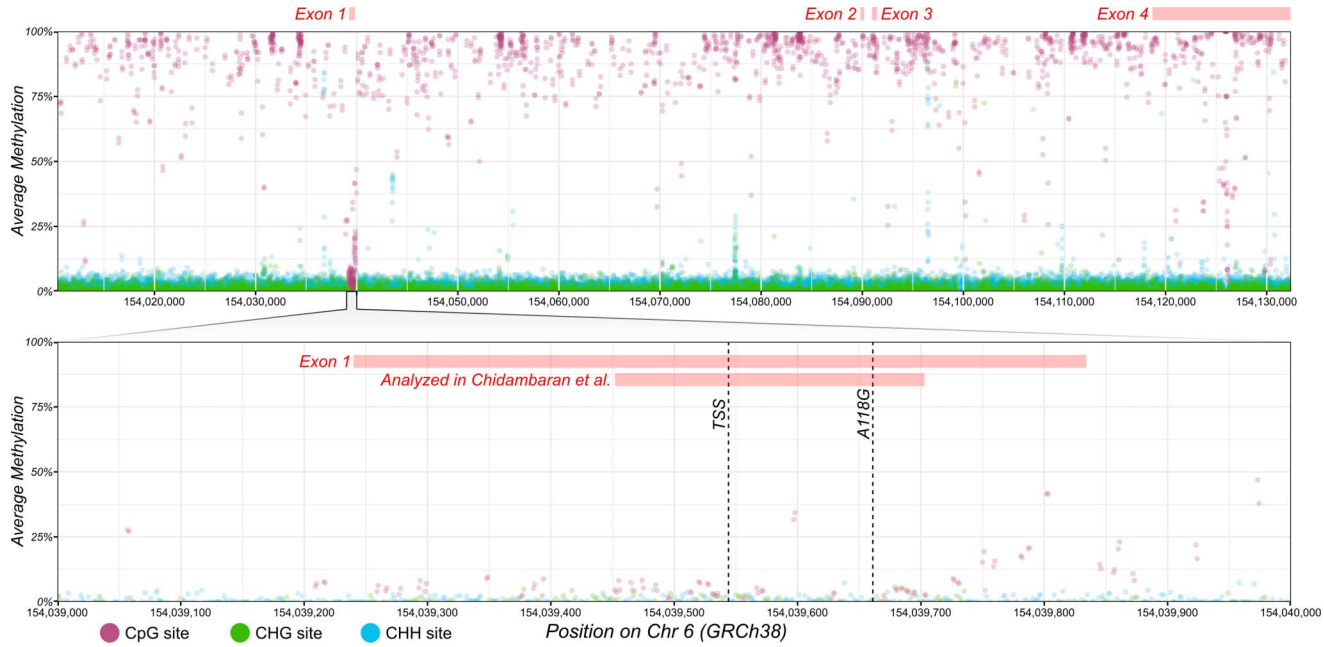

Figure 1. Illustration of *OPRM1* CpG site methylation for selection of CpG sites that were analyzed. Each dot represents the average % methylation (minimum coverage = 4) of CpG (purple), CHG (green), or CHH (blue) individual cytosine sites across genomic regions Chr6:154,000,000-154,130,000 averaged across all samples (N = 112). Bottom panel depicts the dense CpG hypomethylation at exon 1. Red boxes denote locations of *OPRM1* exons as well as the region previously investigated in Chidambaran et al. Dotted lines represent the position of *OPRM1* transcription start site (TSS) and location of rs1799971 (A118G). CpG-specific methylation at Chr6:154,039,209-154,039,803 was chosen for subsequent analyses.

significant CpG sites and *OPRM1* and also identified molecules consistently involved in pathways surrounding 3 major themes: immune cell proliferation/differentiation, inflammation, and respiration. For example, molecules identified to interact between TFs and *OPRM1* for the 216-217 CpG site included estrogen receptor 1, which is known to directly interact with CEBPA, CEBPA and indirectly with CEBPG and *OPRM1*. Furthermore, many of these identified molecules were involved in the "inflammatory response" pathway (Supplementary Fig. 3, available at http://links.lww.com/PR9/A255).

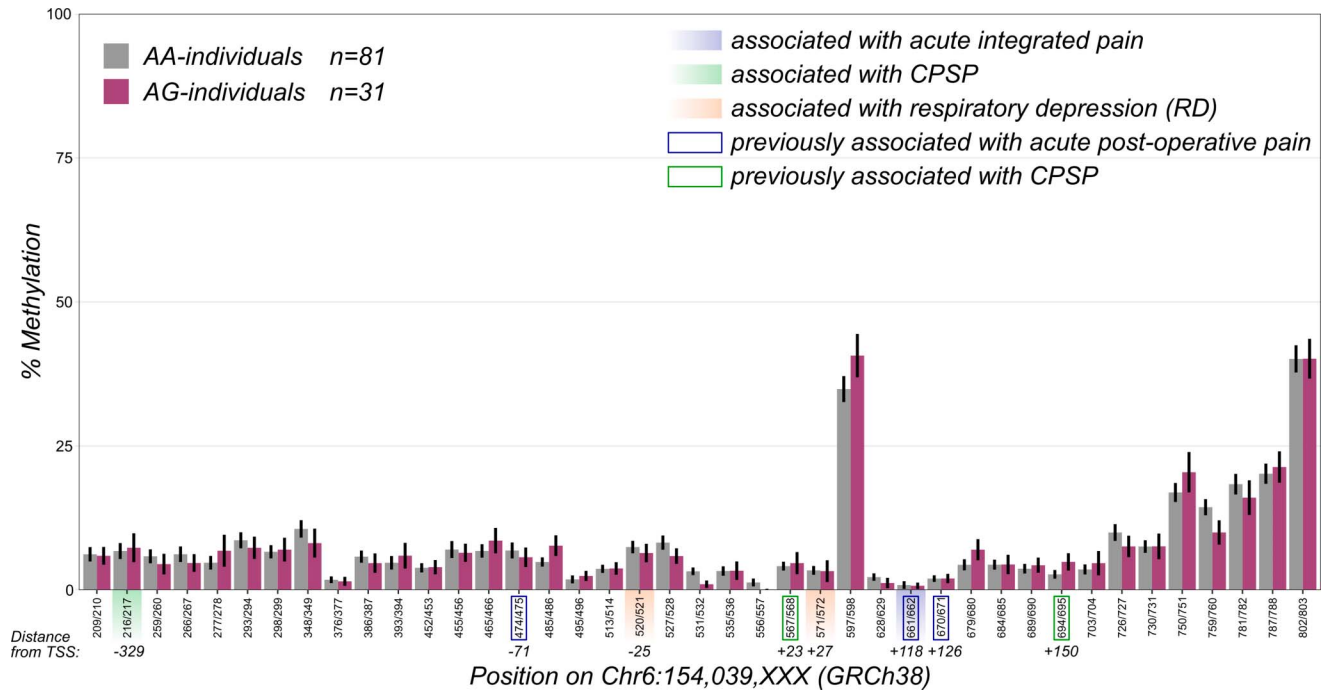

Figure 2. Average methylation at *OPRM1* promoter CpG sites by A118G genotype. AA-individuals (gray) and AG-individuals (purple) from Chr6:154,039,209-154,039,803. All locations beyond 545 are part of *OPRM1* exon 1. Distance from the transcription start site (TSS) is indicated below the axis. CpG sites that are associated with acute integrative pain, chronic postsurgical pain (CPSP), and respiratory depression (RD) in this study or in our previous study, Chidambaran et al., are highlighted accordingly. No significant differences ($P < 0.05$) were found between genotypes. Error bars denote ±SEM.

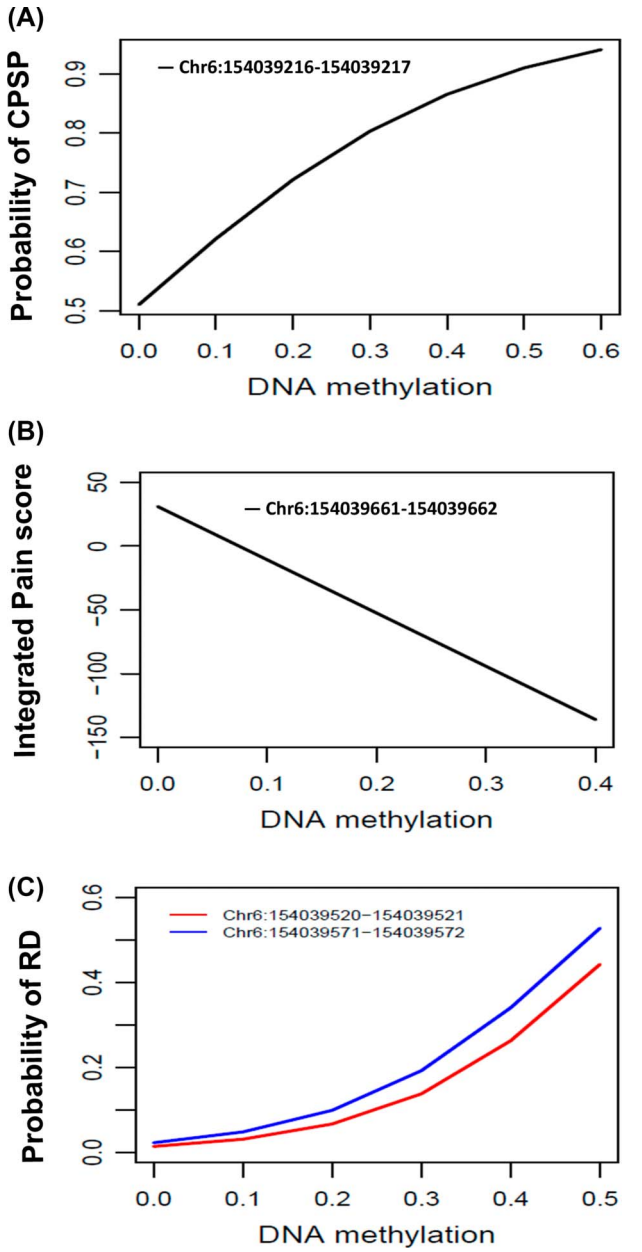

Figure 3. Model predicted predictions outcome by %DNA methylation at CpG sites. (A) Chronic postsurgical pain (CPSP): Chr6:154039216-154039217, (B) integrated pain score: Chr6:154039661-154039662, and (C) respiratory depression (RD): Chr6:154039520-154039521 and Chr6:154039571-154039572. DNA methylation % is shown on *x*-axis and determined by the range of the actual DNAm data.

4. Discussion

4.1. Statement of findings, strengths, and limitations

To investigate the roles of both *OPRM1* DNAm and the most commonly studied variant rs1799971 in determining opioid analgesic efficacy, safety, and CPSP in children undergoing major surgery, we recruited 112 pediatric patients undergoing PSF surgery or pectus excavatum repair and followed them over 12 months after surgery. We conducted genotyping and enzymatic methylation sequencing on presurgical blood samples and examined reads overlapping *OPRM1*. The study design has advantages of generalization, by including patients from multiple regions across the United States and 2 different types of major musculoskeletal surgeries and accounting for multimodal aspects of pain management. A methodological advantage of the current study was the utilization of enzymatic conversion of DNA. Compared to bisulfite sequencing, enzymatic conversion of DNA is less harsh and produces libraries with more uniform GC coverage, and greater mapping efficiency with sensitive detection of both 5-mC and 5-hmC.[56] Since pain is a biopsychosocial phenotype, we also included sociodemographic and psychosocial variables in our analyses making it comprehensive.

We found that DNAm at CpG site 216-217 (promoter) was positively associated with CPSP. However, contrary to our hypothesis, we found decreased acute integrated pain with DNAm at CpG site 661-662, located just past the TSS, within exon 1; of note, Chr6:154039662 is the CpG site introduced by the A118G variant, rs1799971. Finally, our hypothesis of DNAm being associated with lower side effects was not supported, as there was a higher likelihood of RD with DNAm at CpG sites 571-572 and 520-521 in those receiving higher MEq/kg. We found no significant genotype associations with DNAm and no genetic–epigenetic interactions on outcomes. This is likely because no homozygotes for the SNP were identified in our cohort, despite the variant satisfying Hardy Weinberg equilibrium, as the expected frequency of GG in White populations is low. Other study limitations include small sample size and low NG EM-seq coverage.

4.2. DNA methylation associations and functional relevance

Of the 4 CpG sites associated with outcomes (**Table 3**), 1 was located inside the promoter region of *OPRM1*, and the rest were immediately past the TSS in the region of the first exon. Epigenome-wide analyses previously identified DNAm in regions downstream of promoter regions, past the TSS in the region of the first exon, to show stronger transcriptional silencing than DNAm upstream near the promoter regions of genes.[8] The association between increased CpG DNAm at site 661-662 and lower integrated pain observed in the current study is different from pyrosequencing study results in a PSF-only cohort where Chr6:154039661 was positively associated with acute pain in children.[16] We also confirmed that acute pain was also negatively associated with DNAm at this site. Nevertheless, association of DNAm at CpG site 661-662 with acute pain responses after major musculoskeletal surgeries in children is now supported by 2 distinct methylation analytic techniques. We previously found that CpG DNAm at positions 567 and 694, downstream of the TSS, was positively associated with CPSP.[16] Here, we found that DNAm at CpG site 216-217, immediately before the TSS, was associated with CPSP with 1.26 higher odds per 5% increase in CpG DNAm. Association of increased pain with increased DNAm at *OPRM1* promoter regions upstream of exon 1 has also been found in adults with chronic musculoskeletal pain compared to controls, where 2 CpG sites located at chr6:154,009,292 and chr6:154,009,356 exhibited 7.7% increased methylation in cases compared to controls.[50] Thus, despite differences across studies or the exact regions analyzed, DNAm at *OPRM1* promoter regions significantly affects CPSP/chronic pain.

DNA methylation can cause transcriptional activation[65] or repression[31] by binding of TFs, or conversely, TF-binding can affect DNAm.[27] Furthermore, different TF families are known to differ in their responses to CpG methylation; basic leucine zipper members generally do not bind to their motifs when methylated, while members of the extended homeodomain family tend to have increased binding to their motifs when methylated.[65] We postulate that these differential effects of TF domain binding

could be another reason why some directional incongruities were observed compared to our hypothesis. For example, TFs of the homeodomain family were predicted to bind at CpG 661-662 and 571-572 where DNAm was associated with lower integrated pain and increased RD, respectively (higher opioid effects), while TFs of the basic leucine zipper family was predicted to bind at CpG 216-217, whose DNAm was associated with increased CPSP (potentially gene repression). Of interest, these CpG sites were in Polycomb repressive regions, which are involved in development and differentiation, maintaining gene silencing, possibly by inhibiting TF-mediated activation of transcription.[6] Genes with CpG island promoters repressed by Polycomb have been shown to be more likely to be silenced (in other conditions such as cancer).[25,40] The molecules elucidated by our IPA generated network could be targets of future research on mechanisms of opioid effects and pain outcomes.

4.3. Sociodemographic factors

Female sex, psychosocial factors (PPST), and medications (acetaminophen and dexmedetomidine) were found to be associated with pain outcomes, consistent with previous findings.[38] Biological sex differences in opioid responses have been previously demonstrated in association with *OPRM1* methylation and A118G genotype, in chronic pain.[1] We included race and DI as covariates but did not find associations with outcomes, likely due to less racial diversity. Of note, race was found to be inconsistently associated with post-surgical pain outcomes in children.[32,38,46] Chronic pain differences by race[2] as well as socioeconomic status may affect DNAm,[11] including *OPRM1* DNAm. Thus, future studies are warranted in racially diverse populations, considering social determinants of health and population stratification using genetic ancestry.

4.4. Genotype negative findings

OPRM1 rs1799971 occurs in approximately 11% to 37% of the population with population frequencies varying greatly by race.[51] It functions as a hypomorph possibly altering ligand affinity[5] and reducing protein expression/availability.[61] *OPRM1* expression is believed to be reduced by increased promoter methylation[39] and decreased glycosylation resulting in a shorter protein half-life.[28] In our study, we found that despite the A118G variant introducing a potential new CpG site, this site was predominantly hypomethylated in G-carriers, contrary to expectation. Since the variant was not associated with pain/opioid outcomes, our findings suggest that differences in outcomes were likely not due to DNAm associated with the variant itself. We also did not find this variant to be associated with increased methylation at other loci within the promoter, similar to previous studies.[50] The effect of rs1799971 polymorphism on CpG methylation may be brain-region and opiate-use dependent, with enhanced effects in homozygous carriers. For example, homozygous GG opiate users had increased methylation compared to opiate naïve individuals in somatosensory and thalamus brain regions, with decreased *MOR* mRNA levels.[39] Also, in Han Chinese, rs1799971 was identified as an methylation quantitative trait locus associated with increased DNAm at 2 CpG sites in the *OPRM1* promoter.[66] This might reflect the higher A>G mutation found in Asians. Of note, we had previously found presence of the G allele in this SNP to be associated with higher pain scores when our cohort included 1 homozygous variant carrier.[13]

Absence of A118G effects on pain/opioid outcomes is not too surprising. Our findings are aligned with studies not showing associations with pain outcomes.[30,33,60,67] While some meta-analyses have found that G allele carriers require more opioids for cancer-related or postsurgical pain,[30] others did not find a consistent association between *OPRM1* 118A>G and post-surgical or neuropathic pain.[57,59] Our prior meta-analysis of genotype associations with CPSP also did not find significant associations with this variant.[12]

5. Conclusions

This comprehensive study found that DNAm at *OPRM1* promoter and near TSS was associated with variable pain and opioid responses, after adjusting for psychological, perioperative, sociodemographic factors, showing promise for preoperative methylation profiling for risk stratification for CPSP and pre-emptive pain optimization.[48] Our results do not support *OPRM1* A118G for risk prediction or as an methylation quantitative trait locus in White populations with low GG frequency.[59]

Disclosures

V. Chidambaran, L. J. Martin, and H. Ji are named inventors on US Patent number 11,618,924 B2 dated April 4, 2023 for "Methods and compositions relating to OPRM1 DNA methylation for personalized pain management" (CCHMC). The remaining authors have no conflicts of interest to declare.

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Author contributions: B. A. Upton and K. N. Krolick conducted data analyses/interpretation, wrote the initial draft of the paper, prepared tables/figures, reviewed, and helped finalize the manuscript. X. Zhang and V. Pilipenko conducted data analyses, visualization, and interpretation; they reviewed and edited the manuscript. L. J. Martin and H. Ji helped conceptualize the analytic plan and study design, supervised data analyses and interpretation, and reviewed and edited manuscript. S. Glynn conducted study recruitment and sample collection, reviewed, and edited the manuscript. A. Ganesh, C. L. Monitto, L. M. Einhorn, and R. J. Ramamurthi were involved in project administration and investigation at other surgical sites, reviewing and editing the manuscript. V. Chidambaran conceptualized the study, coordinated the study conduct including recruitment, data

collection, data analyses, interpretation and revised and finalized the manuscript.

Data availability statement: Data will be added to the Sequence Read Archives in dbGaP NIH portal (study ID 52248) for use for health/medical/biomedical purposes under data use modifiers—nonprofit use only with institutional review board approval.

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