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1 # Effect of short-term prescription opioids on DNA methylation of the
2 # *OPRM1* promoter

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13 ## Abstract

14 **Background.** Long-term opioid use has been associated with hypermethylation of the opioid
15 receptor mu 1 (*OPRM1*) promoter. Very little is currently known about the early epigenetic
16 response to therapeutic opioids. Here we examine whether we can detect DNA methylation
17 changes associated with few days use of prescribed opioids. Genome-wide DNA methylation
18 was assayed in a cohort of 33 opioid-naïve participants who underwent standard dental surgery
19 followed by opioid self-administration. Saliva samples were collected before surgery (visit 1),
20 and at two postsurgery visits at 2.7 ± 1.5 days (visit 2), and 39 ± 10 days (visit 3) after the
21 discontinuation of opioid analgesics.

22 **Results.** The perioperative methylome underwent significant changes over the three visits that
23 was primarily due to postoperative inflammatory response and cell heterogeneity. To
24 specifically examine the effect of opioids, we started with a candidate gene approach and
25 evaluated 10 CpGs located in the *OPRM1* promoter. There was significant cross-sectional
26 variability in opioid use, and for participants who self-administered the prescribed drugs, the
27 total dosage ranged from 5–210 morphine milligram equivalent (MME). Participants were
28 categorized by cumulative dosage into three groups: <25 MME, 25–90 MME, ≥90 MME. Using
29 mixed effects modeling, 4 CpGs had significant positive associations with opioid dose at 2-tailed
30 p-value < 0.05, and overall, 9 of the 10 *OPRM1* promoter CpGs showed the predicted higher
31 methylation in the higher dose groups relative to the lowest dose group. After adjustment for
32 age, cellular heterogeneity, and past tobacco use, the promoter mean methylation also had
33 positive associations with cumulative MME (regression coefficient = 0.0002, 1-tailed p-value =
34 0.02), and duration of opioid use (regression coefficient = 0.003, 1-tailed p-value = 0.001), but

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35 this effect was significant only for visit 3. A preliminary epigenome-wide association study
36 identified a significant CpG in the promoter of the RAS-related signaling gene, *RASL10A*, that
37 may be predictive of opioid dosage.
38 **Conclusion.** The present study provides evidence that the hypermethylation of the *OPRM1*
39 promoter is in response to opioid use, and that epigenetic differences in *OPRM1* and other sites
40 are associated with short-term use of therapeutic opioids.
41

42 **Key words:** prescription opioids, DNA methylation, addiction, opioid use disorder,
43 epigenetics

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44 **Background**

45 Prescription opioids were once considered as a relatively benign treatment for pain
46 management [1, 2]. However, over the past decade, prescribed analgesics have emerged as a
47 major socio-environmental factor that has contributed to the opioid epidemic [3, 4]. For many
48 individuals who develop opioid use disorder (OUD), the initiation phase may begin with
49 treatment for acute pain or minor surgery, with primary care physicians and dentists
50 accounting for a large fraction of prescribed opioids [5-11]. Even short-term use (e.g., up to
51 three days) is a risk factor for some individuals, and the risk for addiction increases
52 proportionally with dosage and duration of use [8, 9, 12-15].

53 Drug addiction is a chronic disease that is triggered by an exposure to an environmental agent.
54 Following the initial exposure, the addictive substance continues to have a persistent effect,
55 and this suggests a form of cellular memory. There is strong evidence that epigenetic processes,
56 including DNA methylation, play a key role in maintaining the long-term effects of the additive
57 substance [16, 17]. Studies particularly in model organisms have shown that drugs of abuse
58 trigger intracellular signaling cascades that alter gene transcription; repeated exposure to the
59 drug then results in remodeling of the epigenome that persists over time; and these epigenetic
60 processes maintain the long-term changes in steady-state gene expression that underlie
61 addiction [16, 18-20]. Work in humans generally relies on postmortem tissue from long-term
62 drug users, and studies have found significant epigenetic differences in brains of former addicts
63 compared to non-addicts [21, 22]. While the brain is the most relevant tissue in terms of
64 neuroadaptation and drug seeking behavior, epigenetic markers of addiction have also been
65 detected in peripheral tissues such as blood and sperm [23-28]. Easily accessible peripheral

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66 tissues are clearly the practical choice when it comes to defining biomarkers of drug use and/or
67 predictors of individual risk for addiction.

68 The μ-opioid receptor gene (*OPRM1*) encodes the primary target for both endogenous and
69 exogeneous opioids and plays a central role in mediating the rewarding and therapeutic effects.
70 The CpG island located in the promoter of this gene is a potential sensor for drug use, and
71 multiple studies in leukocytes and sperm have found higher DNA methylation among long-term
72 opioid users compared to control samples [23, 29-33]. Hypermethylation of the promoter
73 region has also been found among people with alcohol dependence [34]. However, as all these
74 studies are cross-sectional comparisons between opioid-exposed individuals and controls, there
75 is no definite way to discern whether the epigenetic differences are the cause, or effect, of drug
76 use. Since genetic variants both within, and near the *OPRM1* gene have also been associated
77 with susceptibility to addiction and drug sensitivity[35-37], it is plausible that such epigenetic
78 markers represent genetic effects that preceded drug use. Another lingering question is, if the
79 epigenetic changes are induced by drug use, does the hypermethylation of the promoter CpGs
80 occur only after repeated and sustained exposure, or are these indicators of the early
81 epigenomic, and potentially transcriptomic, responses to drugs? In the case of potent drugs
82 such as opioids, the initial exposure is a crucial phase in the pathway to drug dependence and
83 addiction, and it is reasonable to expect that some of the modification to the epigenome occurs
84 within the first few exposures.

85 To address these questions, we applied a longitudinal design and collected saliva samples and
86 self-reports of opioid use from a group of opioid naïve dental patients before oral surgery, and
87 at two follow-up visits after surgery. We assayed genome-wide DNA methylation and explored

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88 (1) the methylome during the perioperative period, (2) how demographic variables such as age
89 and race/ethnicity relate to methylome changes and immune response, and (3) whether we can
90 discern opioid associated CpGs from the highly heterogeneous methylome data. As the site of
91 surgery and postsurgery inflammatory response, the saliva presents particular challenges due
92 to immune-related cellular heterogeneity. To overcome this, we applied *in-silico* approaches to
93 deconvolute the underlying cellular heterogeneity and demonstrate the utility of the
94 methylome-based cell estimates as proxies for the immune changes induced by surgery. For the
95 effect of opioids, we specifically focused on the *OPRM1* promoter CpGs and evaluated whether
96 the data replicates the CpG hypermethylation. Overall our results show a dose-dependent
97 increase in methylation at the *OPRM1* promoter that can be discerned despite extensive
98 heterogeneity in the methylome data, and this indicates that the epigenetic response to opioids
99 occurs within the first few days to weeks following drug exposure. Additionally, we also
100 performed an epigenome-wide association study (EWAS), and this identified a few CpGs that
101 may be predictive of opioid dosing.

102 **Results**

103 The number of enrolled participants (N = 41) and timeline of sample collection are shown in Fig.
104 1. Only 33 patients (19 females) received prescription opioids after an oral procedure. The
105 baseline characteristics, other diagnosed diseases, casual use of other drugs (specifically
106 tobacco and marijuana; no participant reported use of cocaine, psychedelics, and other hard
107 drugs), and prescription opioid self-administration are reported only for these 33 participants
108 (Table 1). Following the pre-surgery visit (visit 1 or v1), the second visit (visit 2 or v2) occurred
109 after surgery and within a week of the last opioid dose (average number of days between last

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110 opioid dose and visit 2 was 2.7 ± 1.5 days). The last sample collection (visit 3 or v3) occurred
111 between 32–88 days from surgery, and the number of days between the last opioid dose and
112 visit 3 was 39 ± 10 days. In total, 26 participants provided saliva samples at all three visits, 6
113 participants provided saliva at two visits, and one provided saliva only at v1 (Table 1). The mean
114 age was 33.61 ± 13.84 years and ranged from 19 to 61 years (Table 1). Based on self-reported
115 race/ethnicity, there were 13 Caucasians (mean age = 31.69 ± 14.11 years), 13 African
116 Americans (mean age = 39.92 ± 13.91), and the remaining 7 were of "other" racial/ethnic group
117 (mostly Hispanic/Latino; mean age = 25.43 ± 8.02). The African American group was slightly
118 older but there was no statistically significant difference in age between the groups (p-value =
119 0.06). Sex distribution was not significantly different between the race/ethnic groups. Individual
120 level information, including comorbidities, is provided as Additional file 1: Table S1.

121 Postoperative opioid dosing data was based on self-reported pill counts converted to morphine
122 milligram equivalent (MME). With the exception of one individual who used no opioids (and we
123 considered this individual to represent a dose of 0 MME with 0 days of use), all patients started
124 opioid treatment generally within 24 hours of surgery, and continued use for an average of 6 ±
125 4 days for up to 17 days (Additional file 1: Table S1). As expected, cumulative dosage correlated
126 with length of use ($r = 0.67$, p-value < 0.0001). For the 32 participants that self-administered
127 opioids, the total cumulative dosage over the course of treatment ranged from 5–210 MME.
128 Based on the quantile distribution of the cumulative MME, participants were classified into
129 three groups: <25 MME (those below the 25th percentile or quartile 1 for opioid dosage) , 25–90
130 MME (those within the interquartile range), and ≥90 MME (those above the 75th percentile or
131 quartile 3) (Table 1). Opioid dosage showed no significant association with age, sex, and self-

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132 reported race/ethnicity. There was no significant association between opioid dosage and the
133 presence or absence of other comorbidities. Dosage was also not associated with past
134 marijuana use. However, the group that reported using tobacco within the past 12 months had
135 significantly higher self-administered opioid dosage (mean of 95.63 ± 57.78 MME among
136 tobacco users, and 49.55 ± 36.19 MME among non-users; p-value = 0.008).

137 **Table 1. Participant characteristics and postoperative opioid use**

Variables[a]	
Sex	
Female	19
Male	14
Age (years)	33.61 ± 13.84
Self-reported race/ethnicity	
African-American	13
Caucasian	13
Other[b]	7
Tobacco in past 12 months[c]	
Yes	11
No	22
Marijuana in past 12 months[c]	
Yes	10
No	23
Other disease diagnosis[c]	
Yes	17
No	16
Prescribed opioid medication	
Hydrocodone 5mg	19
Oxycodone 5mg	12
Oxycodone 10mg	1
Oxycodone 5mg and Codeine 30mg	1
Length of opioid use in days (mean ± sd)	6 ± 4
MME[d] **(mean ± sd)**	64.91 ± 48.88
<25 MME (<Q1)	10
25–90 MME (Q1–Q3)	13
≥90 MME (≥Q3)	10
Surgery to visit 2 in days (mean ± sd)	8.0 ± 4.1
Surgery to visit 3 in days (mean ± sd)	43.9 ± 10.9
Number of completed visits	
Three visits (v1, v2, and v3)	26 participants

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Two visits (v1 and v2)	5 participants [e]
Two visits (v2 and v3)	1 participant
Only v1	1 participant

138 [a] Mean and standard deviation (sd) for continuous variables and counts for categorical variables
139 [b] Other= Hispanic/Latino, Asian, Middle-eastern, and Native American
140 [c] Self-reported data on other drug use and diagnosis of other diseases (diseases listed in Additional file 1: Table S1)
141 [d] Opioid dose converted to morphine milligram equivalent (MME) according to medication type; Q1 is the first
142 quartile (25%) and Q3 is the third quartile (75%)
143 [e] Methylome data for one participant with v1 and v2 samples were excluded during the methylome data check
144 (see methods)

145

146 Global shift in postoperative methylome

147 For an overview of the methylome and the variance structure, we started with a principal
148 component analysis (PCA) using the full set of high quality probes (736,432 probes passed QC
149 criteria). The top PC (PC1) captured a vast portion of the variance at 63.5%, and following that,
150 PC2 and PC3 captured only 2.5% and 1.6% of variance, respectively (PCs for each methylome
151 data in Additional file 1: Table S2). PC1 was not significantly associated with the demographic
152 variables (sex, age, self-reported race/ethnicity), or with comorbidities and past use of
153 marijuana or tobacco. Instead, visit was the most significant explanatory variable for PC1 ($F_{2,86}$=
154 5.94, p-value = 0.004), and the pattern indicated a significant change in the methylome with the
155 strongest contrast between v3 and v2 (Tukey-Kramer *post hoc* p-value = 0.003) (Fig. 2a). To
156 deduce whether the longitudinal variance capture by PC1 could be explained by the length of
157 time from surgery or opioid self-administration, we performed bivariate analyses between PC1
158 and the following variables: opioid dose, days from surgery to sample collection, and days from
159 last opioid self-administration to sample collection. This analysis was done for the three visits
160 separately, and at v2, PC1 had a modest but significant correlation with days from surgery to v2
161 (r = 0.40, p-value = 0.03, n = 31 participants with methylome data at v2; Fig. 2b). Similarly, at v3,

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162 PC1 was correlated with days from surgery to v3 (r = 0.41, p-value = 0.04, n = 27 participants
163 with methylome data at v3). PC1 was not correlated with opioid dose or the number of days
164 from the last opioid use. From this, we can infer that the longitudinal shift in the methylome is
165 primarily due to surgery.

166 To profile the CpGs that changed longitudinally over the three visits we performed a mixed
167 effects ANOVA with visit as a fixed variable and the person ID as random effect (Fig. 2c). The p-
168 values for visit showed a significant deviation from the null hypothesis (Fig. 2d histogram).
169 However, only 2 intergenic CpGs (cg05639411 and cg24904009) were above the genome-wide
170 significant threshold of 5.0e-8 (Fig. 2c) and overall, the pattern indicated a modest shift in the
171 methylome across several CpGs. At a genome-wide suggestive threshold of p-value = 1.5e-5,
172 there were 1701 CpGs that underwent change over the visits (Additional file 1: Table S3). The
173 majority of these CpGs (>65%) decreased in methylation between v1 and v2, and regained
174 methylation by v3 such that these sites showed significantly higher levels of methylation at v3
175 compared to both v1 and v2 (Fig. 2e). Similarly, for the ~35% of CpGs that gained methylation
176 between v1 and v2, these sites generally declined in methylation by v3 resulting in significantly
177 lower methylation compared to both v1 and v2 (Fig. 2e). Gene set enrichment analysis (GSEA)
178 of the 1133 annotated genes represented by the CpGs conveyed mostly an innate immune
179 inflammatory response (Additional file 1: Table S4). The most overrepresented pathway was
180 natural killer cell mediated cytotoxicity (KEGG ID hsa04650; normalized enrichment score = -
181 1.93, FDR = 0.03), and the most overrepresented function was for genes involved in cellular
182 defense response (GO ID 0006968; normalized enrichment score = -1.83 p = 0.001, FDR = 0.3),
183 and these immunity-related categories were enriched among the CpGs that decreased in

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184 methylation at v2. The opioid receptors were not represented in the list of visit associated
185 CpGs. Based on these observations, a possible explanation for the shift in the methylome is that
186 it is the result of surgery-induced immune response and changes in the oral cell composition.
187 Opioid use, if it had an impact, is likely to exert a weaker signal, and given the limited sample
188 size,more suitable for a focused candidate gene study.

189 Deconvolution of cellular heterogeneity

190 To decompose cell types from the composite DNA methylation signal, we applied a reference-
191 free approach [38]. The bootstrapping method described in Houseman et al. [38] determined K
192 = 4 cell types (Additional file 1: Table S2). Cell 1, which represented the most abundant cell
193 type, showed an increase at v2 right after surgery followed by a decline by v3 (Table 2). Aside
194 from cell 1, no other cell showed significant change over the visits (Table 2). To deduce what
195 cell types are represented by the 4 groups, we also estimated blood leukocyte proportions
196 (mainly lymphocytes and granulocytes/neutrophils) using a reference-based approach [39], and
197 compared correlations between the 4 cell types to the reference-based cell estimates (Table 2;
198 Additional file 1: Table S2). Cell 1 had a strong positive correlation with granulocytes, and cell 4
199 had a strong positive correlation with lymphocytes indicating that cells 1 and 4 are chiefly
200 representative of the leukocyte population in saliva, and serves as a proxy for the increase in
201 granulocyte proportions after surgery. Cells 2 and 3 had only modest correlations with
202 leukocyte estimates (|r| of 0.4–0.5) and may be more representative of the epithelial cells.

203 The cell estimates were not associated with opioid dose. To evaluate if the baseline
204 characteristics were related to cellular composition, we tested associations with age, sex, and

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205 race/ethnicity. Cell 1 had a significant negative correlation with age (r = -0.48, p-value = 0.006)
206 only at v2 that suggests an age-dependent immune response in the days immediately after
207 surgery (Fig. 3a). Cell 3 had the strongest association with age at all three visits (Fig. 3b). Cells 2
208 and 3 showed extensive cross-sectional variability without longitudinal change, and both were
209 significantly associated with race/ethnicity at all three visits, indicating that these could serve as
210 proxies for the cellular composition differences between populations (Fig. 3c, 3d). Cell 4 was
211 not associated with any of the baseline variables, and sex was not a factor for any of the cell
212 types.

213 **Table 2. Reference-free and reference-based estimates of cellular proportions**

	Cell proportions by visit (mean ± SD)				Pearson r with reference-based estimates	
Cell types	**Visit 1**	**Visit 2**	**Visit 3**	**Visit p-val**	**Lymphocytes**	**Granulocytes**
		Reference-free estimates				
Cell 1	0.60 ± 0.28	0.74 ± 0.23	0.47 ± 0.32	$F_{2,86}$ = 6.6, 0.002	-0.96	0.95
Cell 2	0.13 ± 0.13	0.10 ± 0.10	0.17 ± 0.15	ns	0.51	-0.47
Cell 3	0.21 ± 0.20	0.12 ± 0.18	0.22 ± 0.19	ns	0.43	-0.40
Cell 4	0.07 ± 0.18	0.03 ± 0.08	0.14 ± 0.23	$F_{2,86}$ = 2.7, 0.07	0.77	-0.81
		Reference-based estimates				
Granulocytes	0.71 ± 0.14	0.77 ± 0.10	0.64 ± 0.18	$F_{2,86}$ = 6.1, 0.003		
Lymphocytes	0.27 ± 0.12	0.21 ± 0.09	0.33 ± 0.15	$F_{2,86}$ = 6.6, 0.002		

214

215 Effect of opioid dose on *OPRM1* promoter methylation

216 To examine if higher opioid dose is related to higher promoter methylation, we started with a
217 candidate gene approach and focused on the CpGs located in the *OPRM1* promoter. In total, 10
218 promoter CpGs were targeted by the Illumina probes and these encompassed the CpG island
219 described by Nielsen et al. and replicated by Chorbov et al. [29, 30] (Fig. 4a; Table 3; individual

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220 level β-values in Additional file 1: Table S2). We first applied a mixed regression model with
221 opioid dosage group and visit as fixed categorical variables, and each participant ID as random
222 intercept. With the exception of the last CpG, the regression estimates for all the *OPRM1*
223 promoter CpGs were positive, with higher methylation levels for the two higher dosage groups
224 (i.e., 25-90 MME and ≥ 90 MME) relative to the lowest dosage group (<25 MME) (Table 3). At a
225 nominal p-value of 0.05, 4 CpGs were significantly associated with opioid dosage groups. The
226 ANOVA plots for these CpGs showed that the difference between dosage groups was
227 pronounced at v3 (for CpG1, CpG2, CpG6) and v2 (for CpG7) but not at v1 (Fig. 4a). As tobacco
228 use was associated with higher self-administered dosage of opioids, we considered it as a
229 potential contributing factor. However, including past tobacco use in the regression model did
230 not alter the results, and this indicated that the higher methylation at the *OPRM1* CpGs is a
231 specific effect of opioids.

232 To check whether the association with opioid dosage can be robustly detected, we summarized
233 the overall methylation pattern in the promoter by taking the mean DNA methylation β-values
234 for the nine CpGs that were positively associated with opioid dosage (CpG1 to CpG9). We
235 applied a linear regression model and tested whether higher mean methylation was associated
236 with either higher MME or longer length of opioid use. This analysis was done for the three
237 visits separately and adjusted for age, tobacco use, and cellular heterogeneity. Both MME and
238 length of opioid use were associated with higher mean methylation, but this effect was
239 significant only at v3, further indicating that the hypermethylation of the *OPRM1* receptor is
240 more likely a response rather than a predisposing factor (Fig. 4b; Table 4). Our results are
241 consistent with the opioid associated hypermethylation and indicates that even a relatively

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242 short-term opioid use may induce an increase in methylation that is proportional to dosage at

243 the *OPRM1* promoter.

244

245 **Table 3. Dose dependent methylation of individual *OPRM1* promoter CpGs**

		<25 MME vs. 25–90 MME[1]		<25 MME vs. ≥90 MME[1]		Dosage anova[2]	
CpG	**ProbeID**	**Coef**	**t-val**	**Coef**	**t-val**	$F_{2,29}$	***p***
CpG1	cg22370006	0.041	2.66	0.023	1.39	3.53	**0.04**
CpG2	cg14262937	0.051	2.77	0.014	0.69	4.20	**0.02**
CpG3	cg06649410	0.047	1.65	0.010	0.31	1.56	0.23
CpG4	cg23143142	0.018	1.56	0.000	-0.04	1.71	0.20
CpG5	cg23706388	0.010	0.76	0.006	0.38	0.29	0.75
CpG6	cg05215925	0.019	2.73	0.012	1.59	3.74	**0.04**
CpG7	cg14348757	0.042	2.78	0.019	1.16	3.92	**0.03**
CpG8	cg12838303	0.026	2.08	0.022	1.64	2.38	0.11
CpG9	cg22719623	0.006	0.52	0.004	0.33	0.14	0.87
CpG10	cg15085086	-0.029	-0.94	-0.040	-1.19	0.78	0.47

246 [1]Regression estimates for higher dose groups (25–90 MME and ≥90 MME) relative to lowest dose group (<25
247 MME) based on linear mixed effects model: lmer(CpG ~ dose + visit + (1|ID))
248 [2] Two-tailed p-values for the main effect of dosage groups
249

250 **Table 4. Mean methylation in the *OPRM1* promoter and association with opioid dose and**

251 **days of use**

	MME dosage effect Visit 3[2]			Days of opioid use effect Visit 3[2]		
	Coef	**t-val**	***p* (1-tailed)**	**Coef**	**t-val**	***p* (1-tailed)**
Promoter methylation [1]	0.0002	2.16	0.02	0.003	3.40	0.001

252 [1]*OPRM1* promoter methylation summarize by averaging the β-values for CpG1 to CpG9
253 [2]Linear regression at visit 3, one-tailed p-value to test hypermethylation with higher cumulative MME or longer
254 duration of use

255 Preliminary epigenome-wide association study for opioid dose

256 Since the candidate gene approach indicated that the short-term use of prescribed opioids can

257 have an impact on CpG methylation, we expanded the analysis to an EWAS using the same

258 mixed model to test association with opioid dosage. A CpG (cg08105965) located in the

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259 promoter CpG island of the GTPase signaling gene, *RASL10A* (RAS like family 10 member A), was
260 genome-wide significant (p-value of 5.0e-8; Fig. 5a). Unlike the pattern for the *OPRM1*
261 promoter, the lowest dose group had significantly lower methylation level at both visits 1 and
262 3, indicating that the difference preceded opioid use (Fig. 5b). In addition to the *RASL10A*
263 promoter CpG, 5 other CpG sites were associated with opioid dosage at the suggestive
264 threshold (p-value of 1.0e-5; Fig. 5c–g). For most of these, the methylation differences were
265 apparent at v1 and preceded opioid use. For these top CpGs, adjusting for past tobacco use did
266 not alter the results, and none of these sites were significantly associated with tobacco use. To
267 explore if any of the CpGs that were above the suggestive threshold have been previously
268 implicated in opioid use or dependence, we referred to recent human EWAS for opioid
269 dependence [24], and methadone treatment dosage [28]. Based on comparison of probe IDs
270 and genes, none of the CpGs we report here have been previously linked to opioid related
271 traits.

272 While this is preliminary results from a small study cohort and is yet to be replicated, we
273 provide the list of 64 CpGs that were associated with opioid dosage at a nominal uncorrected p-
274 value of 1.0e-4, along with the gene ontology IDs and KEGG pathways for the corresponding
275 genes in Additional file 1: Table S5.

276 Discussion

277 Here we report results from a longitudinal study of DNA methylation in a cohort of opioid naïve
278 dental patients who received prescription opioids following oral surgery. To summarize the
279 main result, we found increased methylation at the *OPRM1* promoter associated with higher

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280 cumulative opioid dose. This replicates the hypermethylated profile among long-term opioid
281 users and alcohol dependent individuals [23, 29-34]. The pattern of methylation we observed
282 indicates that the increase in methylation is more likely the response to, rather than the cause
283 of, opioid use [20]. The present study provides evidence that such epigenetic modifications are
284 induced within the early days of drug use and may represent early epigenomic responses to an
285 addictive substance.

286 A peculiar challenge we faced was that the site of sample collection was also the site of surgery.
287 Saliva has a highly heterogeneous cellular makeup and is estimated to constitute about ~45%
288 epithelial cells, and about ~55% leukocytes from circulating blood [40]. The main goal of the
289 study was to detect the effect of short-term and comparatively low-dose opioids, while
290 accounting for the larger perturbation caused by surgery. Although we do not have details on
291 the severity of the oral surgery, most were third molar extractions and were relatively minor
292 and non-invasive. Nonetheless, the patients would have experienced an injury-induced
293 inflammatory response that can result in changes in numbers of circulating immune cells [41],
294 and consequently, changes in oral cell composition. As DNA methylation is highly cell-type
295 specific, the heterogeneity in cells will be a major source of "noise" in the methylome data [39,
296 42-44]. The longitudinal variability in DNA methylation that was captured by the top PC can
297 therefore be attributed to cell composition rather than opioid use. We could deduce this by the
298 significant correlation between PC1 and the number of days from surgery to the follow-up
299 visits. We were able to partly resolve the cell heterogeneity by applying reference-free and
300 reference-based estimates of cell proportions. The reference-free method estimated four major
301 cell types. Although saliva is highly heterogeneous, and certainly has more than just 4 types of

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302 cells [40], the classification into 4 broad groups likely reflects the limitation in the *in-silico*
303 approach to resolve finer differences between cellular subtypes. Cell 1 most likely represented
304 the granulocyte population (chiefly neutrophils), which constitutes the most abundant
305 leukocyte subtype in circulating blood, and is responsible for innate immunity and acute
306 inflammatory response. Consistent with the known increase in granulocyte-to-lymphocyte ratio
307 in the few days following surgery [45], we also found an increase in cell 1 and in relative
308 abundance of granulocytes compared to lymphocytes at visit 2. This was followed by a
309 compensatory decrease in granulocyte proportions by visit 3. Cell 2 and cell 3 are presumed to
310 represent a portion of the epithelial cell population, and these showed no significant within-
311 individual changes over the visits. However, these cells exhibited significant association with
312 age and self-reported race/ethnicity. Although cell type decomposition was not the primary
313 objective of the study, our analyses demonstrated that the saliva methylome can be highly
314 informative of individual differences in perioperative immune profiles.

315 For the effect of postsurgical opioid use, we first focused on the *OPRM1* promoter region as an
316 epigenetic sensor of opioid dose. The CpG-rich promoter harbors a CpG island and several
317 studies in different populations have demonstrated higher DNA methylation at this site among
318 opioid users and methadone-maintained heroin addicts [23, 29-33]. The increased methylation
319 of the *OPRM1* promoter is not only limited to OUD but has also been detected among
320 individuals with alcohol dependence, suggesting that the hypermethylation is generally
321 associated with substance use disorder and addiction [34]. A question has been whether such
322 epigenetic differences are the result of drug use or the cause of increased vulnerability to
323 addiction [20]. To address this, we interrogated 10 CpGs in a 550 bp region that encompassed

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324 the promoter CpG island investigated by Nielson et al. and Chorbov et al. (the CpG island is
325 depicted in Fig. 4) [29, 30]. With the exception of the last CpG, the remaining 9 CpGs showed
326 higher methylation in the two higher-dose groups relative to the low-dose group, and four of
327 these CpGs were significantly associated with dosage at nominal alpha of 0.05. Comparison of
328 mean methylation differences between the dosage groups across the three visits indicated that
329 higher methylation in the higher dose groups is more apparent at the postsurgery visits,
330 particularly visit 3. The positive association between the mean promoter methylation and
331 cumulative MME, and mean promoter methylation and days of opioid use, were also significant
332 only at visit 3. The heightened inflammatory state at visit 2, which occurred within a few days of
333 surgery, may have been the reason why the more subtle effect of opioids was not significant at
334 visit 2, and the positive association emerged only at visit 3.

335 The *OPRM1* locus presents a prime site for gene x environment interaction, a critical aspect of
336 addiction since the addictive substance is an environmental agent that has a long-lasting
337 biological effect. The *OPRM1* gene has been the subject of several candidate gene studies for
338 addiction. Much attention has been paid to the missense SNP that alters the *OPRM1* protein
339 function, although its impact on addiction traits and OUD is somewhat ambiguous [46, 47].
340 Several studies have also identified non-coding variants in the *OPRM1* locus that alters DNA
341 methylation and gene expression [33, 35, 48]. At least one genome-wide association study has
342 also identified a genome-wide significant association between a SNP upstream of *OPRM1* and
343 methadone-maintenance dosing [37]. These studies collectively provide evidence that common
344 genetic variants in the proximal region of *OPRM1* affect DNA methylation and gene expression,
345 and could have a downstream impact on opioid response that could potentially influence

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346 vulnerability to addiction. Our present work was carried out in a small sample size and our
347 primary goal was to track the within-individual trajectory across visits. If there were genetically
348 modulated small cross-sectional differences at baseline, this sample size would be
349 underpowered to detect the differences, and the significant association with opioid dose that
350 we found may have been the result of opioid-induced augmentation of differential methylation
351 at the postoperative visits.

352 The hypermethylation of the *OPRM1* promoter is likely only a small part of a larger network of
353 genes involved in the cellular response to drug exposure. We therefore followed up with a
354 preliminary EWAS exploration to identify other CpGs that may be associated with opioid self-
355 administration. To our surprise, despite the small sample size, one CpG, located in the promoter
356 region of *RASL10A*, a Ras-related GTPase signaling gene, was genome-wide significant. Perhaps
357 this is due to the power of the longitudinal design in capturing differentially methylated sites
358 that are significantly different between dosage groups at more than one visit. For instance, the
359 differential methylation of cg08105965 at *RASL10A* is apparent at both visits 1 and 3. Although
360 *RASL10A* has not been previously implicated in opioid response or addiction, it is notable that
361 the OPRM1 protein is a G-protein coupled receptor, and its activation results in cellular
362 signaling cascades that also involve Ras GTPase activity [49-51]. In addition to the *RASL10A* CpG,
363 five other CpGs were at or above the suggestive threshold, including sites located in *AFF1*,
364 *VSNL1*, *ANXA2*, and *PAIP2*. To our knowledge, DNA methylation at these genes have not been
365 previously linked to opioid use or dependence. However, one recent study of gene expression
366 in the rat model has shown an upregulation of *RASL10A* and *VSNL1* in the brain following acute
367 morphine treatment [52]. Similar to *RASL10A*, *VSNL1* (visinin-like 1) also codes for an

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368 intracellular signaling molecule with high expression in the brain [53]. The list of CpGs that were
369 associated with opioid dosage at a nominal uncorrected p-value < 0.0001 included few other
370 cellular signaling genes (e.g., *ANXA2*, *RET*, *ADRB1*). Taken together, the EWAS results hint that
371 epigenetic modulation of genes involved in intracellular signal transduction may play a role
372 during the early phase of opioid use.

373 We must emphasize that the small sample size and the heterogeneity in methylome signal,
374 partly due to cell composition and partly due to the heterogenous population group, are major
375 limitations, and the EWAS results await replication in an independent cohort. The CpGs
376 identified by the present EWAS were differentially methylated even at v1, prior to opioid use,
377 and this suggests that there may be genetic variants underlying these epigenetic differences.
378 However, such potential effects of genetic variation is not addressed in the present study due
379 to the lack of genotype data. Another weakness that we should note is that the main variable of
380 interest, therapeutic opioid dosing, was based on patient self-reports rather than objective
381 measures of drug use [54]. A future strategy would be to use existing technologies such as
382 wearable devices that can provide additional means of tracking the physiological responses to
383 opioids [55]. The present study also does not address whether these epigenetic changes linger
384 or diminish over time in the absence of continued drug use. A more comprehensive longitudinal
385 epigenomic study of the early effects of prescription opioids that also integrates genetic effects,
386 and with a longer follow-up period would be the next phase of study.

387 Regarding the potential for epigenetic persistence, we must point out that any peripheral tissue
388 serves only as a proxy for the possible epigenetic changes in the brain. A distinction is that
389 blood and epithelium are mitotically active tissues and cells are renewed within a few days to a

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390 few weeks, with the exception of long-lived memory T-cells. For methylation signals to persist,
391 it will require either continued presence of the perturbation (i.e., continued exposure to
392 opioids), or methylation changes in mitotically active stem cells that can be faithfully
393 transmitted to daughter cells. The brain, on the other hand, is mitotically inactive and consists
394 of mostly terminally differentiated cells that last a lifetime. If the relatively modest dose and
395 short-term use of prescription opioids has a similar impact in brain cells, the effects may not
396 readily decay and may be long lasting in the central nervous system.

397 Conclusion

398 In conclusion, our study replicates the hypermethylation of the *OPRM1* promoter with opioid
399 use. Previous studies reported on the effects of chronic opioid use; here we provide evidence
400 that the epigenetic restructuring begins within the initial stage of opioid exposure. The present
401 findings on the acute effects of prescription opioids, as well as the CpGs that may be predictive
402 of opioid dosing, require further replication with a well-powered and more comprehensive
403 study in a larger cohort.

404 Methods

405 Participants

406 Eligible participants were scheduled for tooth extractions, mostly third molar extractions, at an
407 oral and maxillofacial surgery clinic that were typically followed by postoperative prescriptions
408 of hydrocodone/acetaminophen (7.5/325 mg q4-6h prn pain) or oxycodone/acetaminophen
409 (5mg/325mg q6h prn pain). For inclusion in the study, individuals were required to be 18 years
410 of age or older, opioid naïve, able to consent, able to understand and speak English, and willing

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411 to provide saliva samples. Individuals were excluded if they reported previous use of opioids,
412 had current substance use dependence, were pregnant, were incarcerated, had other causes of
413 pain, were unable to consent, or had a developmental disability that prevented participation.
414 The study received approval by the university Institutional Review Board. Eligible participants
415 were provided a summary of the consent form by the study coordinator and allowed to read
416 and ask questions before enrollment. All participants provided written informed consent.

417 Forty-one individuals consented to the study and provided contact information and responded
418 to a demographic questionnaire. The enrolled participants also provided information on
419 existing diagnosed diseases, and were assessed for casual substance use within the past 12
420 months (tobacco, marijuana, cocaine, psychedelics, other hard drugs; details in Additional file 1:
421 Table S1). The clinical staff provided routine opioid medication and recovery instructions for all
422 participants right after surgery. The opioids prescribed to participants were Hydrocodone,
423 Oxycodone, and Codeine in doses that varied between 5mg to 30mg (Table 1). The study
424 coordinator also provided opioid medication logs to participants to record self-administration
425 including the date, time, individual dose per opioid pill, and number of opioid pills taken. For
426 the 33 participants who received opioid medication, only one participant (person ID 142)
427 reported no opioid usage.

428 **Sample processing and DNA methylation assay**

429 Saliva was collected using the Oragene DNA sample collection kit (OGR 500) by DNA Genotek
430 (http://www.dnagenotek.com). The first set of samples was collected before surgery. The
431 second saliva sample was collected a few days after opioid discontinuation, and the third was
432 collected on a follow-up visit (Fig. 1; Table 1). DNA was purified using the DNA Genotek PrepIT

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433 L2P kit according to manufacturer's instruction. Genome-wide DNA methylation was assayed
434 on the Illumina Infinium Human MethylationEPIC BeadChips following the manufacturer's
435 standard protocol at the HudsonAlpha Genomic Services Lab (https://gsl.hudsonalpha.org).

436 **Data processing**

437 Raw intensity IDAT files were loaded to R and all quality checks, data preprocessing, and
438 normalization were carried out using the R package, minfi (v.1.31) [56]. Methylation levels were
439 estimated as β-values (ratio of methylated by unmethylated probes) and quantile normalized.
440 The initial QC involved comparison between the log median intensities of methylated and
441 unmethylated channels, and the density plots for β-values (Additional file 2: Fig. S1a). All
442 samples passed these checks. Sex estimated from the DNA methylation data also matched the
443 self-reported sex. To retain only high-quality data, probes with detection p-value > 0.01 (14,676
444 probes) were excluded. Probes that target CpGs on the sex chromosomes were also removed
445 (18,605 probes). Finally, a total of 96,146 probes that overlapped annotated SNPs and/or were
446 flagged for poor mapping quality (MASK.general list from [57]) were also filtered out. A total of
447 736,432 high quality probes were retained and used for downstream analysis.

448 As further QC, we performed unsupervised hierarchical clustering using the full set of high-
449 quality probes (Additional file 2: Fig. S1b). While samples longitudinally collected from the same
450 individual tended to cluster together, there were also several samples that did not cluster with
451 self. To check for possible errors in sample labeling, we repeated the cluster analysis using a
452 subset of 30,435 probes that had been filtered out due to overlap with common SNPs. While
453 these were deemed poor-quality probes and unfit for differential methylation analysis, in terms

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454 of sample identity check, these probes can serve as proxy genotype markers that can help verify
455 if samples came from the same person. Using this set, almost all samples collected from the
456 same participant clustered with self, and for the most part, the clusters also aligned with self-
457 reported race/ethnicity groups (Additional file 2: Fig. S1c). Only one of the 33 participant who
458 received prescription opioids (person ID 108) did not pair with self and data from this person
459 were excluded from all downstream analysis.

460 **Estimation of cellular proportions**

461 To infer the relative proportions of the major cell types, we first implemented a reference-free
462 deconvolution of the methylome data using the R package RefFreeEWAS (v2.2) [58]. The
463 RefFreeEWAS algorithm applies a non-negative matrix factorization to decompose a matrix Y =
464 MΩ, where M represents an $m \times K$ matrix with m as CpG specific methylation for an unknown
465 number of K cell types, and Ω as the cell-type proportion constrained to sum to a value ≤ 1. For
466 computational efficiency, the K cell types has to be first specified, and as described in
467 Houseman et al. [58], we set the K to vary from 2 to 10 cell types and decomposed the Y = MΩ.
468 Following this, we applied bootstrapping to estimate the optimal K value. For this estimation,
469 we applied 10 iterations with replacement every 1000 times. The optimal $K = 4$ was selected
470 based on the minimum value of the average of bootstrapped deviances for each putative cell
471 type. While this method provides the relative proportions of cell types, the identity of the four
472 cells are unknown. Since a significant proportion of saliva consists of leukocytes, we also
473 applied a reference-based approach to estimate the relative proportions of lymphocytes and
474 neutrophils [39, 42, 43]. To infer the putative identities of cells, we performed Pearson
475 correlations between the K cells and the proportions of leukocyte types.

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476 **Statistical analyses**

477 For the global analysis, PCA was done on the full set of 736,432 probes using the prcomp
478 function in R. In order to evaluate which variable had the most significant association with PC1,
479 we examined the association between PC1 and the following variables: sex, age, self-reported
480 race/ethnicity, presence or absence of other diseases, tobacco or marijuana usage in the past
481 12 months, and opioid dose, days from surgery to sample collection, and days from last opioid
482 dose to sample collection. We used ANOVA for categorical variables, and Pearson correlation
483 for continuous variables; and these tests were conducted separately for the three visits. We
484 also performed similar analyses for estimated cell proportions to examine whether the
485 variables were significantly related to the cell proportions. PC1 and the cell proportions were
486 also related to visit using ANOVA. Since visit was the most significant explanatory variable for
487 PC1, we identified the CpGs that showed longitudinal change over the three visits by applying a
488 mixed-effects ANOVA: aov(β-value ~ visit + Error(ID/visit)). This epigenome-wide analysis was
489 done for the 26 participants with data from all 3 visits. For the set of genome-wide suggestive
490 CpGs that changed over the visits (uncorrected p-value $\leq 10^{-5}$), GSEA was implemented on the
491 WebGestalt platform (http://www.webgestalt.org) with each CpG ranked by the mean β-value
492 difference between v2 and v1.

493 For candidate gene analysis, we surveyed the promoter region of *OPRM1*. The CpG island that
494 was interrogated by Nielson et al., and Chorbov et al. is located at 154360587–154360922 bp of
495 chromosome 6 (GRCh37/hg19) [29, 30]. Within that exact coordinate, our data only had 4 CpG
496 probes. We therefore considered a slightly wider region (550 bp) and in total, the array data
497 contained 10 probes that targeted promoter CpGs at chr6:154360344-154360894 bp. To

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498 evaluate methylation at individual CpGs, we applied a linear mixed-effects model with dosage
499 group and visit as fixed categorical variables, and person ID as random intercept: lmer(β-value ~
500 dosage + visit + (1|ID)). To test if history of tobacco use could account for some of the effects,
501 we repeated the test with the model: lmer(β-value ~ MME + tobacco-use + visit + (1|ID)). This
502 was done using the "lmertest" R package, and to get the p-values for the main effect of dosage
503 groups, the degrees of freedom were computed by the Satterthwaite's method [59, 60].
504 Following the CpG level analysis, we estimated the general methylation trend for the promoter
505 by averaging the β-values for the 9 CpGs that had a positive regression coefficient with the
506 dosage groups. We then tested the association between the promoter mean methylation score,
507 and two opioid-related continuous variables: length of opioid use in days, and cumulative MME.
508 This analysis was done for the three visits separately, and adjusted for age and cellular
509 heterogeneity using the equations lm(mean-β ~ MME + tobacco-use + age + cell1 + cell2 +
510 cell3), and lm(mean-β ~ days-of-use + tobacco-use + age + cell1 + cell2 + cell3).

511 Following the candidate gene study, we then performed an EWAS for opioid dosage using the
512 same mixed-effect model: lmer(β-value ~ MME + visit + (1|ID)); and for the CpGs identified by
513 the EWAS at above the genome-wide suggestive threshold, we checked for the effect of past
514 tobacco use using the model: lmer(β-value ~ MME + tobacco-use + visit + (1|ID)) .

515 List of Abbreviations

516 EWAS: Epigenome-wide association study

517 FDR: False discovery rate

518 GO: Gene ontology

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519 GSEA: Gene Set Enrichment Analysis

520 KEGG: Kyoto encyclopedia of genes and genomes

521 MME: Morphine milligram equivalent

522 OPRM1: Opioid receptor mu 1

523 OUD: Opioid use disorder

524 PC: Principal component

525 PCA: Principal component analysis

526 QC: Quality control

527 SNP: Single nucleotide polymorphism

528 Declarations

529 **Ethics approval and consent to participate:** All participants provided written informed consent
530 and study received IRB approval.

531 **Consent for publication:** Not applicable

532 **Availability of Data.** The full de-identified raw DNA methylation data will be made available
533 from the NCBI NIH Gene Expression Omnibus repository upon official publication.

534 **Competing interests:** We have no financial or non-financial conflicts of interest.

535 **Funding:** Funded by the University of Tennessee Health Science Center CORNET Clinical Awards

536 **Author contributions:** JVSS: performed lab work and data analysis and contributed to
537 manuscript; FISG: contributed to data analysis; JHB: identified suitable patients and facilitated

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538 participant recruitment at the dental clinic; KJD: contributed to study conception and design;
539 KM: contributed to study conception, design and data analysis, and wrote the manuscript. All
540 authors contributed to and approved the final version of the manuscript.

541 Acknowledgements

542 We thank the UTHSC Office of Research and the CORNET Awards for supporting this project.

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718 ## Figure legends

719 **Fig 1. Timeline of sample collection.** Saliva samples were collected before surgery and the two
720 follow-up visits after surgery and end of opioid self-administration. The notations above the
721 arrows show the range of days between events.

722

723 **Figure 2. Global patterns in DNA methylation across visits**

724 **(a)** The top principal component (PC1) extracted from the methylome-wide data explained
725 63.5% of the variance, and the ANOVA plot shows significant differences between the three
726 visits ($F_{2,86}$= 5.94, p-value = 0.004). **(b)** At visit 2, PC1 is correlated with number of days from
727 surgery to the second visit (r = 0.40, p-value = 0.03, n = 31). **(c)** The epigenome-wide association
728 plot depicts the location of each CpG (autosomal chromosomes 1 to 22 on the x-axis) and the –
729 $-\log_{10}$(p-value) for the effect of visit (y-axis). Genome-wide significant threshold was set at p-
730 value = 5×10^{-8} (upper red horizontal line); suggestive threshold was set at p-value = 10^{-5} (lower
731 blue horizontal line). **(d)** Distribution of p-values for the effect of visit shows a significant
732 deviation from the null hypothesis. **(e)** For the CpGs above the suggestive threshold,
733 comparison of mean differences between visit 1 and visit 2 (x-axis), and visit 3 and visit 2 (y-
734 axis) indicates a reversal in methylation patterns from visit 2 to visit 3, with the majority of sites
735 showing lower methylation at visit 2, and then increasing in methylation by visit 3.

736

737 **Figure 3. Estimated cell type proportions and associated variables**

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738 **(a)** Cell 1 shows both longitudinal and cross-sectional variability. Proportion of cell 1 is
739 negatively correlated with age at visit 2 (r = -0.48, p-value = 0.006, n = 31; black squares and
740 dashed line), but not at visit 1 (r = -0.13, p-value = 0.49, n = 31; red x markers and dotted line),
741 and only slightly at visit 3 (r = -0.32, p-value = 0.10, n = 27; grey circles and solid line). **(b)** Cell 3
742 is associated with cross-sectional variability but no significant longitudinal change. The
743 estimated proportion has a strong positive correlation with age at all three visits. At visit 1, r =
744 0.36 (p-value = 0.05); visit 2, r = 0.57 (p-value = 0.0009); visit 3, r = 0.48 (p-value = 0.01). **(c)** Cell
745 3 also shows a significantly higher proportion in African Americans at all three visits ($F_{2,28}$ =
746 15.66, p-value < 0.0001 at visit 1). **(d)** Cell 2 is also ethnicity specific and associated with lower
747 proportion in African Americans at all three visits ($F_{2,28}$ = 4.77, p-value < 0.02 at visit 1).

748

749 **Figure 4. *OPRM1* promoter CpG methylation**

750 **(a)** The *OPRM1* promoter and the CpG island (green block) are depicted along with base pair
751 coordinates (black line; GRCh37/hg19), and location of the 10 CpGs (filled circles). Residual β-
752 values were extracted after fitting participant ID as random intercept, and the plots show the
753 methylation patterns across the three visits for CpG1, CpG2, CpG6, and CpG7 (panels with
754 ANOVA line plots; error bars are standard error). The difference between the dosage groups
755 appears at visit 3 (for CpG1, CpG2, CpG6) and visit 2 (for CpG7). The lowest cumulative dose
756 group (<25 MME: blue dotted line) has lower average methylation compared to the two higher
757 cumulative dose groups (25–90 MME: yellow dashed line; ≥90 MME: red solid line). **(b)** The
758 promoter mean methylation was taken as the average β-values for CpG1 to CpG9. After fitting a

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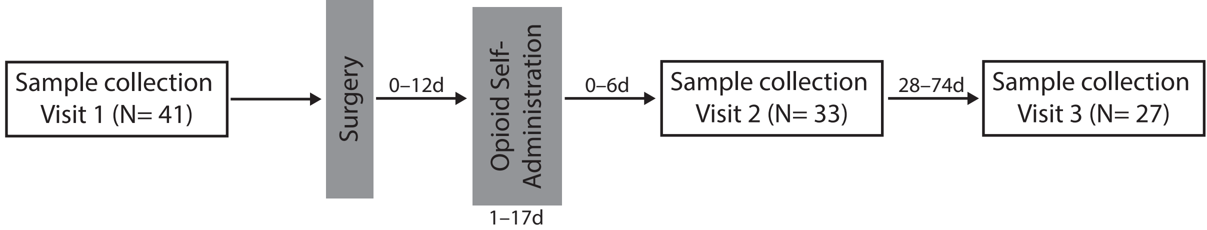
759 regression model with adjustment for age and cell proportions, the leverage plots show a
760 significantly higher average promoter methylation (y-axes) associated with higher MME (x-axis,
761 left panel), and longer duration of use (x-axis; right panel). MME is morphine milligram
762 equivalent.

763 **Figure 5. Epigenome-wide test for prescription opioid dosage.**

764 **(a)** The epigenome-wide Manhattan plot depicts the location of each CpG (autosomal
765 chromosomes 1 to 22 on the x-axis) and the $-\log_{10}$(p-value) for the effect of doage (y-axis).
766 Genome-wide significant threshold was set at p-value = 5×10^{-8} (upper red horizontal line);
767 suggestive threshold was set at p-value = 10^{-5} (lower blue horizontal line).

768 For the six CpG sites that were above the genome-wide suggestive threshold, residual β-values
769 were extracted after fitting participant ID as random intercept, and the plots show the
770 methylation patterns across the three visits (error bars are standard error) for **(b)** cg08105965
771 (*RASL10A*), **(c)** cg18500286 (*AFF1*), **(d)** cg04718304 (intergenic), **(e)** cg16719801 (*VSNL1*), **(f)**
772 cg08163714 (*ANXA2*), and **(g)** cg25124965 (*PAIP2*). For most of these, the difference between
773 the dosage groups appears at visits 1 and 3.

774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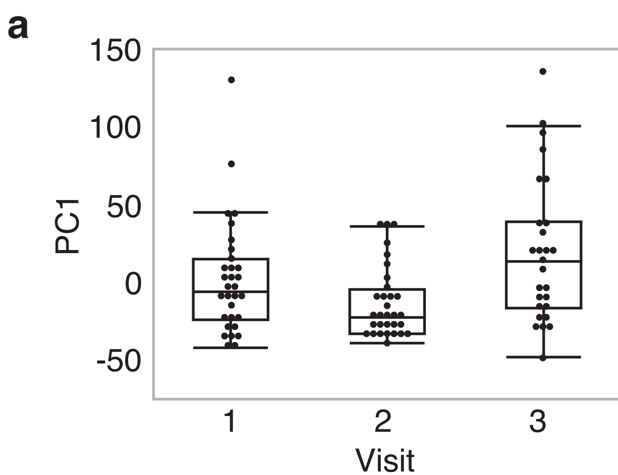
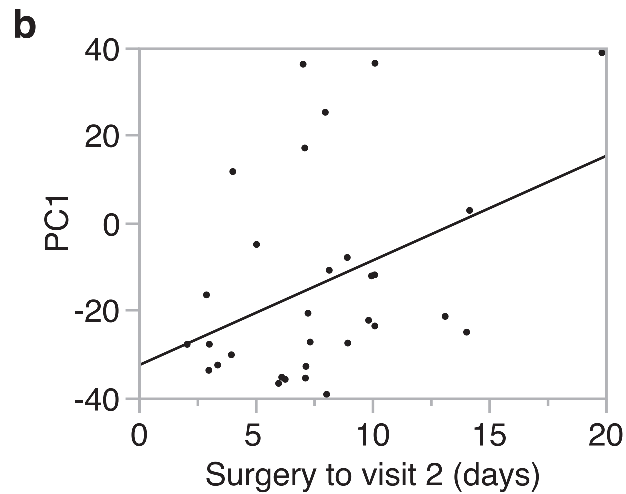
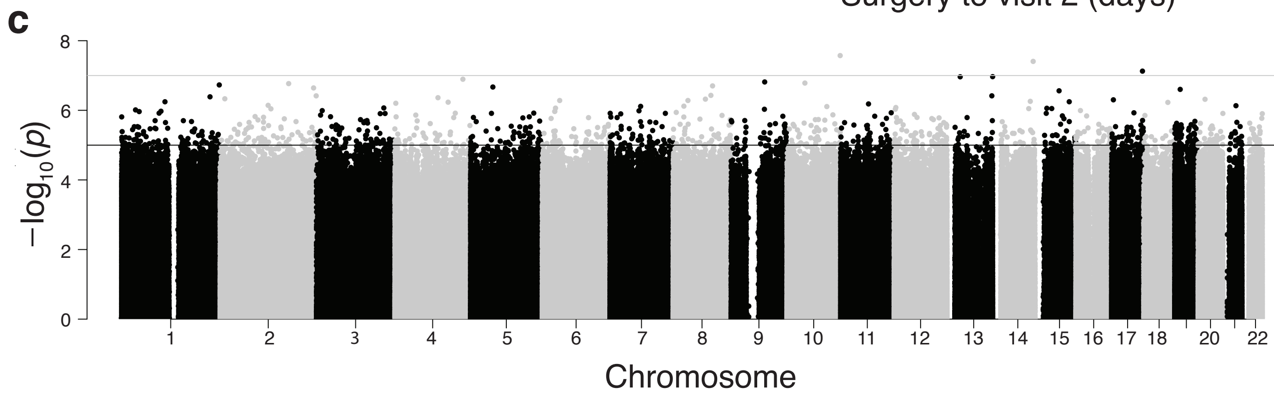
Visit 1 (N= 41)
Surgery
0–12d
Opioid Self-
Administration
1–17d
0–6d
Sample collection
Visit 2 (N= 33)
28–74d
Sample collection
Visit 3 (N= 27)


a
150
100
50
0
-50
PC1
1
2
3
Visit


b
40
20
0
-20
-40
PC1
0
5
10
15
20
Surgery to visit 2 (days)


c
8
6
4
2
0
$-\log_{10}(p)$
1
2
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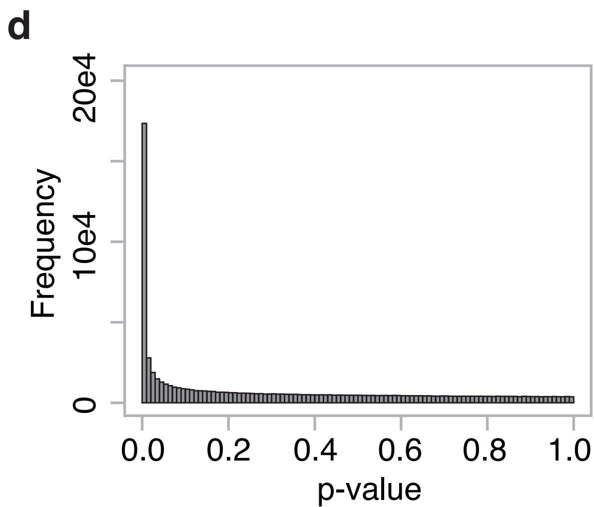
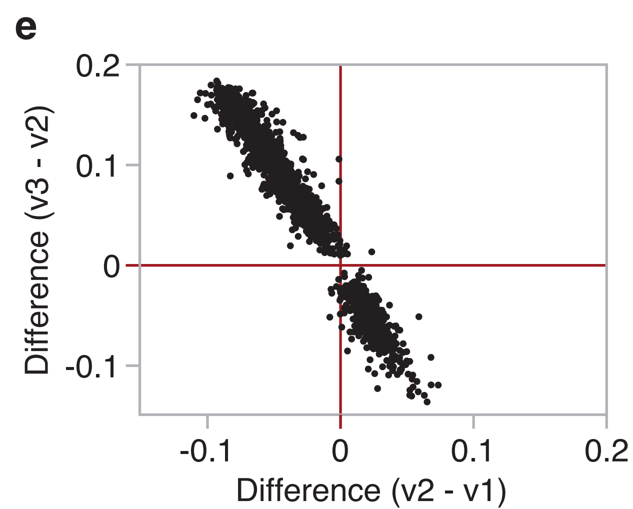
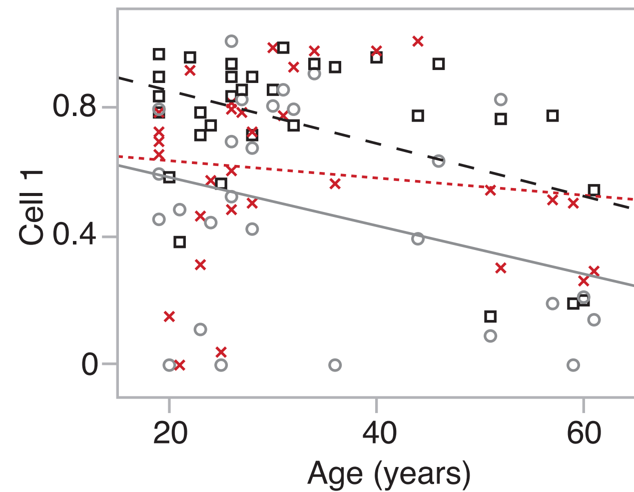
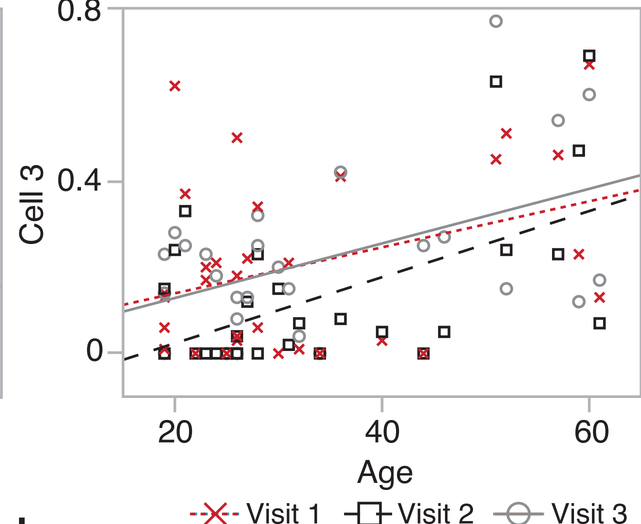
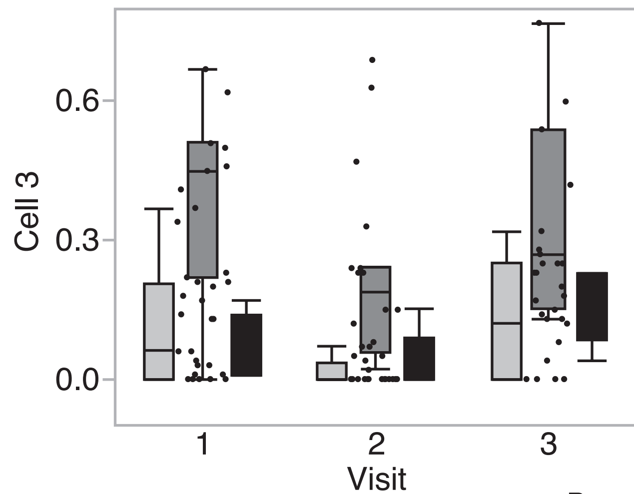
Chromosome

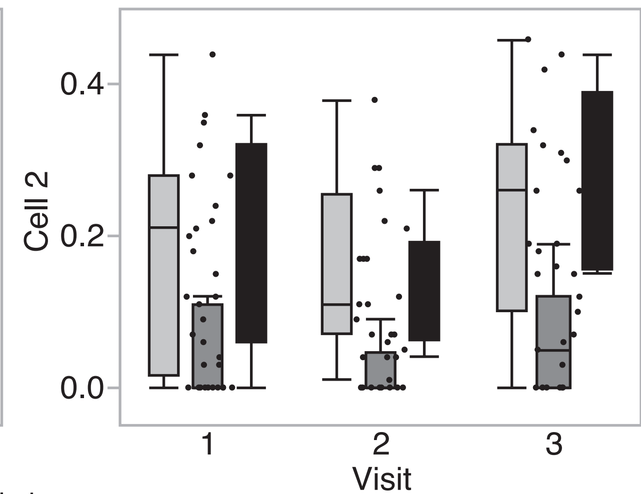

d
20e4
10e4
0
Frequency
0.0
0.2
0.4
0.6
0.8
1.0
p-value


e
0.2
0.1
0
-0.1
Difference (v3 - v2)
-0.1
0
0.1
0.2
Difference (v2 - v1)


a
Cell 1
0.8
0.4
0
20
40
60
Age (years)


b
Cell 3
0.8
0.4
0
20
40
60
Age
Visit 1
Visit 2
Visit 3


c
Cell 3
0.6
0.3
0.0
1
2
3
Visit


d
Cell 2
0.4
0.2
0.0
1
2
3
Visit
Population group
Caucasians/White
AfricanAmericans/Black
Other

a

CpG1 CpG2 CpG6 CpG7

Adjusted β-values

0.02 0.00 -0.02 -0.04

1 2 3

Visit

Dosage

<25 MME 25–90 MME ≥90 MME

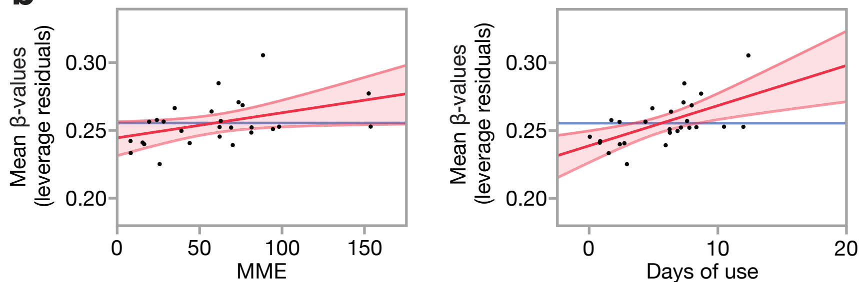
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6 CpG7 CpG8 CpG9 CpG10

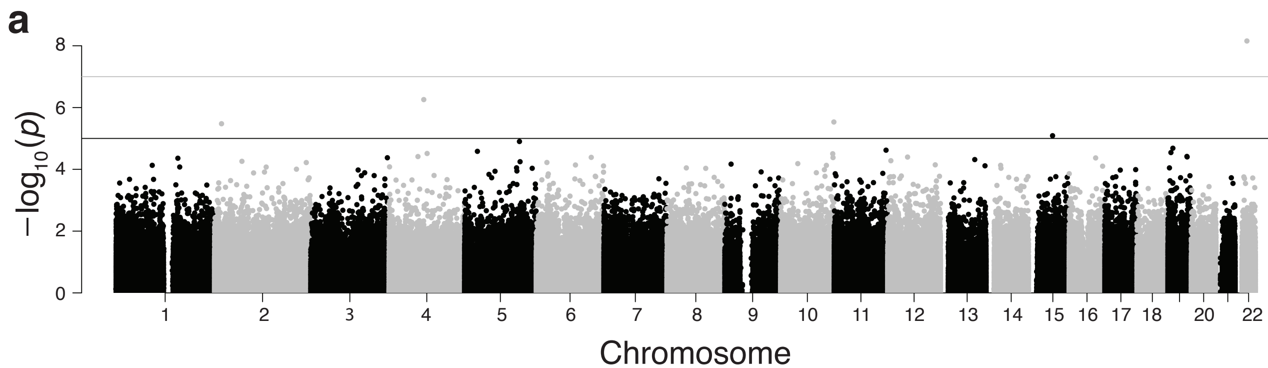
chr6 154,360,200 154,360,400 154,360,600 154,360,800 154,361,000

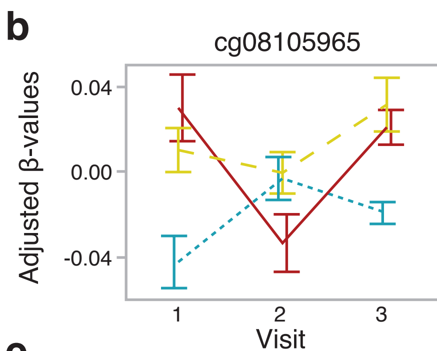
OPRM1

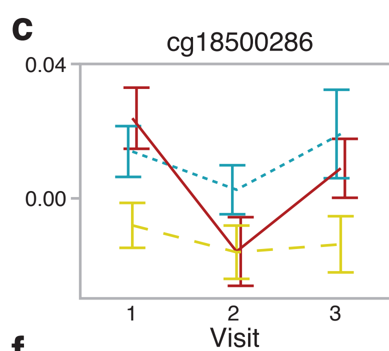
OPRM1 promoter CpGs

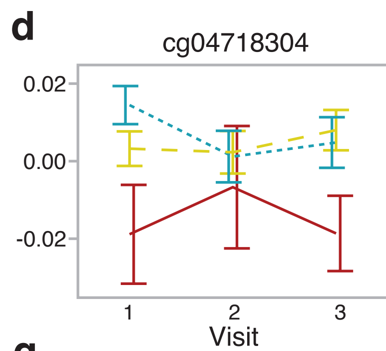
b

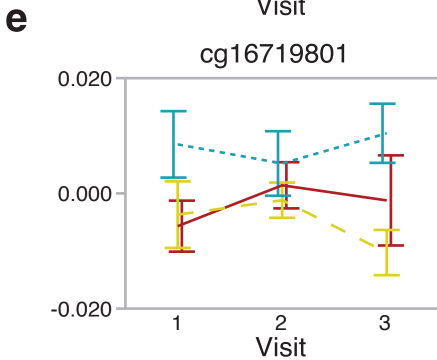


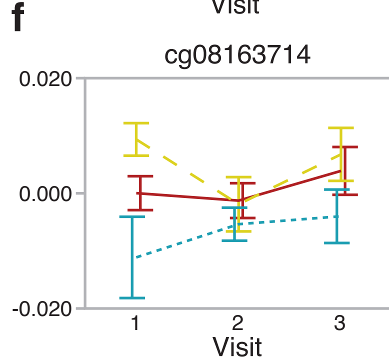


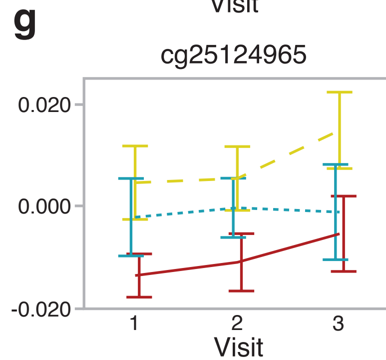












Dosage
<25 MME 25–90 MME ≥90 MME