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Epigenome-wide association analyses of active injection drug use

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ABSTRACT

Background: Injection drug use (IDU) is prevalent in the US and is associated with substantial risk of blood-borne infections, morbidity, and mortality. However, the spectrum of its biologic effects on DNA methylation in blood is not well characterized.

Methods: 401 participants (M_{age} = 47.9; 68% male; 90% African American) over several timepoints (1054 visits) were drawn from a longitudinal cohort of people who inject drugs. DNA methylation was measured among buffy coat samples from the 1054 visits. Compared to samples collected after ≥ 6 months of abstinence, separate EWAS were conducted for active injecting of any drug, quantitative injection frequency, injecting of heroin and injecting of cocaine. Linear mixed effect models were used and analyses were adjusted for repeated measurements and key technical, biological, and sociodemographic characteristics.

Results: We found epigenome-wide significant CpG sites associated with active injection (cg10636246, *AIM2*, $p = 2.33 \times 10^{-8}$) and injection intensity (cg13117953, $p = 4.30 \times 10^{-8}$). We found converging evidence that cg10636246 (*AIM2*), cg23110600 (*PRKCH*), cg03546163 (*FKBP5*), cg04590956 (*GMCL1*), and cg16317961 (*MAPRE2*) were among the top 0.1% significantly differentially methylated CpG sites shared across the five EWAS. Top ranked CpGs among the five EWAS were enriched ($p < 0.0001$) in AIM2 inflammasome complex, T cell migration, insulin regulation and epinephrine synthesis pathways. During periods of active injection, samples had 0.46 years of epigenetic age acceleration relative to the abstinence period, within the same subject ($p = 0.03$).

Conclusions: Findings from this study demonstrate modest, common, and specific effects on DNA methylation during a relatively short time between periods of active drug injection and abstinence.

1. Introduction

The prevalence of illicit drug use is high in the US, with 20.8% of the population reporting past-year use of any illicit substance in 2019, according to the latest results from National Survey on Drug Use and Health (Substance Abuse and Mental Health Services Administration, 2020). In addition, there were an estimated 5.9 million cocaine users, 745,000 heroin users, and 10.1 million opioid misusers (including the misuse of prescription pain relievers) in 2019 in the US (Substance Abuse and Mental Health Services Administration, 2020).

Active injection drug use (IDU) is associated with substantial risk of blood-borne infections, morbidity, and mortality (Bargagli et al., 2001;

Abbreviations: ALIVE, AIDS Linked to the Intravenous Experience; ART, antiretroviral therapy; AUC, Area Under Curve; CpG, cytosine-guanine; EWAS, epigenome-wide association study; FDR, False discovery rate; GO, Gene ontology; IDU, Injection drug use; IQR, interquartile range; PC, Principal component; PWID, people who inject drugs; QQ plot, quantile-quantile plots; SNP, single nucleotide polymorphisms.

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Bird et al., 2003). Studies have shown that people who inject drugs (PWID) have a higher risk of HIV acquisition than the general population (Schoenbaum et al., 1989) and worse HIV outcomes during treatment initiation compared with people who do not inject drugs, primarily due to treatment non-adherence. Moreover, according to a 10-year prospective cohort study in San Francisco, California, the mortality rate among PWID is 10 times greater than the general public (Evans et al., 2012). Among persons who inject drugs (PWID) in Baltimore, MD, the estimated standardized mortality ratio remained elevated over a 10-year period, even after excluding HIV-related mortality (Vlahov et al., 2004), highlighting the need to understand the biological consequences and correlates of IDU.

The exact biological mechanisms of the consequences of active IDU on human health are still unknown. The development of biomarkers indicating these mechanisms has been challenging given the limitations in accessing neural tissues in living patients. Epigenetic markers, especially DNA methylation at cytosine-guanine (i.e., CpG) sites, are common regulators of gene expression (How Kit et al., 2012), and have been strongly associated in blood samples with many diseases such as cancer (Mikeska and Craig, 2014) and environmental exposures such as smoking (Gao et al., 2015). DNA methylation markers open the door to better understanding the biological mechanisms resulting from active drug use, and as a possible peripheral blood biomarker of active IDU. According to recent reports in human blood samples, six CpG sites were significantly associated with IDU and hepatitis C in the promoter regions of the *NLRC5*, *TRIM69*, *CX3CR1*, and *BCL9* genes (Zhang et al., 2017). Another epigenome-wide association study (EWAS) on opioid dependence identified three CpG sites mapping to the *PARG*, *RERE*, and *CFAP77* genes in human whole blood samples (Montalvo-Ortiz et al., 2019). However, the sample size was limited for both studies and there was no within-subject longitudinal examination of DNA methylation profiles during periods of injection drug use and abstinence. Understanding how DNA methylation changes in response to periods of active injecting will help to clarify biological responses to active injection drug use and identify strategies for therapeutic interventions.

In addition to understanding substance-specific associations with DNA methylation, it is valuable to understand the cumulative epigenetic consequences of active IDU. One such indicator of this cumulative effect is epigenetic age. Several epigenetic age calculations exist, including DNA methylation age (Horvath, 2013) and PhenoAge (Levine et al., 2018). Both methods estimate the degree of biological aging based on DNA methylation patterns at CpG sites that correlate highly with chronological age, irrespective of cell and tissue type. PhenoAge is thought to better reflect epigenetic aging in response to specific environmental exposures (e.g., substance use). PhenoAge is calculated using 513 CpG sites (41 of which overlap with DNA methylation age) that are correlated with chronological age and age-related mortality from chronic diseases (Levine et al., 2018). Although developed using whole blood (as opposed to 51 different tissue and cell types in DNA methylation age), PhenoAge correlates strongly with chronological age across tissue and cell types (Levine et al., 2018). Thus, PhenoAge was calculated in the present study to understand epigenetic aging in response to various IDU exposures.

In this study, we used a longitudinal design in the AIDS Linked to the Intravenous Experience (ALIVE) Study to examine the DNA methylation profiles for the same subject during periods of active injection and abstinence. We compared the DNA methylation profiles by active injection and abstinence, injection intensity, heroin injection, and cocaine injection during the past six months. The goal of the study was to further our understanding of active IDU on the epigenome and potentially develop biomarkers reflecting the course of drug use.

2. Materials and methods

2.1. Study sample

The ALIVE Study is an on-going prospective cohort study characterizing the incidence and natural history of HIV infection among PWID in Baltimore, MD, initially established in 1988 (Vlahov et al., 1991). Participants were assessed up to twice annually and the study design has been described in detail previously (Galai et al., 2003; Lambert et al., 2015). At each visit, clinical, behavioral and laboratory data such as HIV infection status and IDU were assessed for the participants. Samples were selected from the entire pool of ALIVE subjects with available buffy coat blood sample and visits discordant for active IDU of any heroin or any cocaine, or abstinence from all recreational drugs. Sampling was based on sequential visits, which occur at a minimum interval of six months, and methylation was measured at each selected visit. To avoid the confounding of order effects due to age, which is strongly reflected in the epigenome, subsets of the data were selected for (1) a transition from active injecting to abstinence (cessation) from one study visit to the next study visit or (2) a transition from abstinence to active injection (relapse). Fig. S1 provides a schematic of our sample. We selected 83 subjects with a transition from active IDU to abstinence then relapse (Type I) and 85 subjects who maintained abstinence at a third visit (Type II). This subsample was selected to examine the impact of continued abstinence on the epigenome. We also selected a set of 127 (Type III) subjects with two visits, transitioning from active IDU to abstinence and an additional set of 69 subjects (Type IV) who transition from abstinence to active IDU. An additional 37 subjects contributed Type III and Type IV transitions across four visits. This yielded a sample from 401 active PWID and 1045 visits among these PWID. The median time between visits where there was a cessation transition was 194 days (interquartile range [IQR]: 182–555), and where there was a relapse transition was 183 days (IQR: 182–234).

The study participants went through HIV serology screening at baseline. HIV serology status was assessed at each study visit for HIV negative participants, whereas CD4+, CD8+ count and HIV viral load testing were performed at each study visit for HIV positive participants. Active injection status, injection intensity, IDU type (i.e., any heroin injection, or any cocaine injection), smoking patterns, and antiretroviral therapy (ART) by face-to-face interview were obtained by computer-administered standardized questionnaires. Injection intensity during the past six months was defined by the following categories and modeled as an ordinal variable: less than once per week, 1–3 times per week, 5–7 times per week, 8–14 times per week and $>$ 14 times per week.

2.2. DNA methylation measurement and preprocessing

DNA was isolated from buffy coat with the Qiagen DNeasy kit and bisulfite converted with Zymo EZ methylation gold kit at the Johns Hopkins University Center for Inherited Disease Research. Bisulfite treated DNA was run on the Illumina Infinium MethylationEPIC BeadChip.

The *minfi* package (Bioconductor) was used to process raw Illumina image files into noob preprocessed methylation beta values (Aryee et al., 2014). Cell composition on CD4+, CD8+, natural killer cells, monocytes, granulocytes, and B cells were estimated based on the method described in Houseman et al. (Houseman et al., 2012) and implemented in *minfi* (Aryee et al., 2014; Jaffe and Irizarry, 2014). Eleven samples with low intensity, inconsistency on predicted and observed sex, or outliers in estimated cell composition were removed for quality control. Probes with low intensity or that are known to cross-hybridize were excluded. A total of 1045 samples and 864,801 probes were used in the final analyses. Batch effects were adjusted for by the top six principal components (PC) from negative control features that are only correlated with technical variations (Gagnon-Bartsch and Speed, 2012). Genetic ancestry was estimated by performing PCA on the 59 single nucleotide

polymorphisms (SNP) probes profiled on the EPIC array, and we retained the top 6 PCs that captured most of the variability.

2.3. EWAS by linear mixed effect model

A linear mixed effect model with random intercepts was used to account for the longitudinal design by using the R package *lme4* and *lmerTest* (Bates et al., 2015; Kuznetsova et al., 2017). M-values were used in the analysis since the M-value distribution is closer to the normality assumption of linear regression (Du et al., 2010).

We conducted single-site EWAS separately for active injecting vs. abstinence, injection intensity, any heroin injection use vs. abstinence, any cocaine injection use vs. abstinence and both heroin and cocaine injection use vs. abstinence during the past six months. The random intercept accounted for variation in DNA methylation levels for each subject. The mixed effect model was adjusted for HIV status, sex, age, smoking methylation score, cell type composition, six negative control PCs and six ancestry PCs. The rationale for using the smoking methylation score is to overcome self-reporting biases on smoking or the impact of second-hand smoking on the methylome. We computed the smoking methylation score using 39 smoking related CpG sites as described in Logue (Logue et al. (2020) and we validated that the smoking methylation score is significantly associated with past six-month smoking status ($p = 3.4 \times 10^{-26}$, Fig. S2). The model is stated below with active injecting as an example (*i* indicates subject and *t* indicate time point):

$$\begin{aligned} M_{it} = {} & u_i + \beta_0 + \beta_1 \text{active injecting}_{it} + \beta_2 \text{HIV}_{it} + \beta_3 \text{sex}_{it} + \beta_4 \text{age}_{it} + \beta_5 \text{smoking methylation score}_{it} \\ & + \beta_6 \text{CD4}_{it} + \beta_7 \text{CD8}_{it} + \beta_8 \text{natural killer cells}_{it} + \beta_9 \text{monocytes}_{it} + \beta_{10} \text{granulocytes}_{it} \\ & + \beta_{11} \text{B cells}_{it} + \beta_{12} \text{negative control PC1}_{it} + \ldots + \beta_{17} \text{negative control PC6}_{it} \\ & + \beta_{18} \text{Ancestry PC1}_{it} + \ldots + \beta_{23} \text{Ancestry PC6}_{it} \end{aligned}$$

Model performance was assessed by quantile-quantile plots (QQ plot) and calculation of the lambda genomic inflation coefficient. Epigenome-wide significance was defined by a false discovery rate (FDR) less than 0.05. The top 0.1% differentially methylated probes and the closest gene for the five EWAS on active injecting, injection intensity, heroin injection use, cocaine injection use and both heroin and cocaine injection use were assessed for overlap by a Venn diagram.

For the purpose of cross-tissue comparison, we evaluated whether the top 10,000 sites overlapped with a recent brain opioid EWAS (Shu et al., 2021). We also conducted sensitivity analyses to account for autocorrelation with the same mixed model adding the first-order autoregressive covariance structure (AR1) by using the nlme package in R (Pinheiro et al., 2017). Another sensitivity analysis on African American samples only (n = 949) was performed with six PCs extracted from the African American samples.

2.4. Gene ontology

The gene ontology enrichment analysis was with CpGs with $p < 1E-05$ using the GO (Gene Ontology) database (Ashburner et al., 2000) in the R package *missMethyl*, with correction for sampling bias due to different numbers of probes per gene (Geeleher et al., 2013; Phipson et al., 2015). Nominal p-values of enriched pathways are presented.

2.5. DNA methylation age

We calculated the PhenoAge (Levine et al., 2018) for all samples based on the DNA methylation profiles and obtained each subject visit's biological age acceleration by taking the difference between PhenoAge and chronological age. We examined the relationship between active injection status and biological age acceleration among 385 subjects with at least one visit where active injection was reported and at least one visit where abstinence from injection drug use was reported. To maximize the time window between active injecting and abstinence within the same person, we selected the first occurrence of active injecting visit in our samples and last occurrence abstinence visit for the biological age acceleration analysis. We used a linear mixed model to assess the association between biological age acceleration and active injection use with adjustment for sex, race, smoking, HIV status and autocorrelation within the same subject by imposing first-order autoregressive covariance structure (AR1). The model is specified as below (*i* indicates subject and *t* indicate time point):

Table 1
Study observation characteristics (n = 1045).

	Unique subjects		All observations	
Sample size	401		1045	
Average age (mean ± se)	47.9 ± 7.6		48.8 ± 7.5	
Average number of visits (mean ± se)	2.6 ± 0.7		2.6 ± 0.7	
Sex				
Male (N, %)	275	68.6%	715	68.4%
Female (N, %)	126	31.4%	330	31.6%
Race				
Non-Hispanic White (N, %)	40	10.0%	96	9.2%
African Americans (N, %)	361	90.0%	949	90.8%
HIV status				
Positive (N, %)	127	31.7%	333	31.9%
Negative (N, %)	274	68.3%	712	68.1%
Past six month active injection status				
Active injecting (N, %)			513	49.1%
Abstinence (N, %)			532	50.9%
Past six month type of injection drug use				
Heroin (N, %)			331	31.7%
Cocaine (N, %)			211	20.2%
Both heroin and cocaine (N, %)			158	15.1%

$$Biological\ Age\ Acceleration_{it} = u_i + \beta_0 + \beta_1 \text{active injecting}_{it} + \beta_2 \text{HIV}_{it} + \beta_3 \text{sex}_{it} + \beta_4 \text{smoking status}_{it} + \beta_5 \text{Ancestry PC1}_{it} + \ldots + \beta_{10} \text{Ancestry PC6}_{it}$$

3. Results

3.1. Sample characteristics

The sample characteristics are shown in Table 1. Among 401 subjects, an average of 2.6 ± 0.7 visits per subject were selected for DNA methylation measurement. In total, DNA methylation from 1045 blood samples were profiled by Illumina Infinium MethylationEPIC BeadChip. 49.1% of the blood samples were extracted during periods of active injecting for the past six months, while 50.9% of the samples were from periods of abstinence for the past six months (Table 1). 405 visits involve any heroin injection use, and 266 visits involve any cocaine injection use, with 196 visits when both heroin and cocaine were injected (Table 1, Fig. S3). Among 401 subjects, there are 31.6% female and 68.4% male participants, and 90% were African Americans and 10% were non-Hispanic White. The average age is 47.9 and the mean age when first used IDU is 21.2. 31.7% participants were HIV positive (Table 1).

3.2. Epigenome-wide association analysis on active injection drug use

The EWAS on active IDU in the past six-months revealed one CpG (cg10636246, *AIM2*) which reached epigenome-wide significance ($\beta = -0.15$, $p = 2.33 \times 10^{-8}$). The top 20 differentially methylated CpGs that were associated with active IDU are listed in Table 2. There was no significant inflation or deflation ($\lambda = 1.11$, Fig. S4a) and the Manhattan plot is shown in Fig. 1**a**. Of note, the CpG sites cg04590956 (*GMCL1*, $\beta = -0.08$, $p = 6.33 \times 10^{-6}$), cg23110600 (*PRKCH*, $\beta = -0.06$, $p = 5.41 \times 10^{-7}$) and cg03546163 (*FKBP5*, $\beta = -0.11$, $p = 2.06 \times 10^{-6}$) were among the top ranked differentially methylated sites.

The EWAS of injection intensity in the past six-months identified one CpG (cg13117953) which reached epigenome-wide significance ($\beta = -0.08$, $p = 4.30 \times 10^{-8}$). The top 20 differentially methylated CpGs that were associated with injection intensity are listed in Table 2. There was no significant inflation or deflation ($\lambda = 0.99$, Fig. S4b) and the Manhattan plot is shown in Fig. 1**b**. The CpG sites cg09356524 (*ZMYND8*, $\beta = 0.03$, $p = 2.19 \times 10^{-6}$) and cg25327296 (*TSPAN18*, $\beta = -0.03$, $p = 1.38 \times 10^{-5}$) were also among the top ranked differentially methylated sites (Table 2). Overall, we identified several significant DNA methylation sites associated with active IDU and injection intensity.

3.3. Epigenome-wide association analysis on heroin injection use and cocaine injection use

We also conducted EWAS on any heroin, any cocaine or both heroin and cocaine injection use during the past six months. The two CpG sites in *AIM2* were among the top ranked differentially methylated sites (cg10636246, $\beta = -0.15$, $p = 2.19 \times 10^{-7}$; cg07195224, $\beta = -0.11$, $p = 2.56 \times 10^{-6}$; Table 3). CpG sites in *PRKCH* and *FKBP5* were also among the top ranked list (cg23110600, *PRKCH*, $\beta = -0.08$, $p = 3.13 \times 10^{-7}$; cg03546163, *FKBP5*, $\beta = -0.13$, $p = 1.45 \times 10^{-6}$) of sites associated with injection of heroin (Table 3). The CpG site cg23110600 (*PRKCH*) was also among the top ranked CpG sites in the EWAS on active injecting ($\beta = -0.06$, $p = 5.41 \times 10^{-7}$, Table 2). No significant inflation or deflation was observed ($\lambda = 1.13$, Fig. S4c). The Manhattan plot is shown in Fig. 2**a**. We also evaluated whether there are any overlapping differentially methylated CpG sites with the brain opioid EWAS (Shu et al., 2021). There are 126 overlapped CpG sites among top 10,000 differentially methylated CpG for blood heroin injection use EWAS and brain opioid EWAS (Table S1). The biological pathways revealed by 89 genes associated with the 126 CpGs and were illustrated by ShinyGO (Ge et al., 2019) (Fig. S5).

There were no epigenome-wide significant CpGs identified in the EWAS of cocaine injection use during the past six-months, however, the CpG sites cg14347670 (*CCND3*, $\beta = -0.14$, $p = 1.87 \times 10^{-7}$) and cg16317961 (*MAPRE2*, $\beta = 0.06$, $p = 8.76 \times 10^{-7}$) was among the top ranked differentially methylated sites (Table 3). No significant inflation was observed ($\lambda = 1.06$, Fig. S4d). The Manhattan plot is shown in Fig. 2b.

3.4. Sensitivity analyses with adjustment for autocorrelation and among African American samples only

We conducted the first sensitivity analyses to see if accounting for autocorrelation would make a difference to our results (Table S2). Compared with EWAS results in Table 2, the top hits among EWAS in active IDU and injection intensity with adjustment for autocorrelation showed similar results, with the same CpG sites in *AIM2*, *PRKCH*, *GMCL1* and *FKBP5* associated with active IDU and CpG sites in *ZMYND8* and *TSPAN18* associated with injection intensity. Similarly, compared with substance specific EWAS results in Table 3, the same CpG sites in *AIM2*, *PRKCH* and *FKBP5* were among the top hits in heroin injection use EWAS after accounting for autocorrelation, and the same CpG sites in *CCND3* and *MAPRE2* were among the top hits in cocaine injection use EWAS.

We also conducted a sensitivity analysis on African Americans only samples (n = 949, Table S3). For active IDU EWAS, cg10636246 (*AIM2*) remains epigenome-wide significant ($\beta = -0.15$, $p = 5.35 \times 10^{-8}$), similar to EWAS results with all samples (Table 2). Other EWAS top hits showed similar results among the same CpG sites with slightly less significance in EWAS with the African American subsamples (Table 2, Table 3, Table S3).

3.5. Gene ontology enrichment analysis

We also conducted gene set enrichment analysis to assess whether top differentially methylated CpG sites are enriched in any known biological pathways. Gene set enrichment analysis showed that CpGs associated with active IDU were enriched in biological pathways such as the 'pyroptosome complex assembly' ($p = 4.73 \times 10^{-4}$), 'response to progesterone' ($p = 1.46 \times 10^{-3}$), 'AIM2 inflammasome complex' ($p = 3.1 \times 10^{-3}$, Fig. 3). CpGs associated with heroin injection use were enriched in positive regulation of T cell migration ($p = 2.99 \times 10^{-4}$) and cellular response to drug ($p = 2.67 \times 10^{-3}$), and CpGs associated with cocaine injection use were enriched in insulin signaling pathways and epinephrine synthesis process (Fig. 4).

3.6. DNA methylation age

We also explored whether DNA methylation age is associated with active IDU. Among visits where no IDU was reported as baseline, the mean biological age acceleration [PhenoAge (Levine et al., 2018) minus chronological age] was − 2.63 years; among visits where active IDU was reported, the difference was closer to zero (−2.00 years), indicating that the biological age acceleration is faster among visits with active IDU compared with visits with no IDU (Fig. 5). After adjusting for sex, race, smoking status and autocorrelation within the same subject, there was a significant 0.46-year difference in biological age acceleration within the

Table 2
Epigenome-wide association analysis of active injection drug use and injection intensity during the prior six months.

Active injection drug use										Injection intensity									
Rank	CpG	Chr	Position	Gene	Relation to Island	Estimate	t value	p value	FDR	Rank	CpG	Chr	Position	Gene	Relation to Island	Estimate	t value	p value	FDR
1	cg10636246	1	159046973	*AIM2*	OpenSea	-0.15	-5.65	2.33E-08	2.02E-02	1	cg13117953	7	157706117		S_Shelf	-0.08	-5.61	4.30E-08	3.72E-02
2	cg04590956	2	70057467	*GMCL1*	Island	-0.08	-5.31	1.46E-07	6.33E-02	2	cg10051222	1	166844628		N_Shore	-0.04	-4.81	2.06E-06	5.72E-01
3	cg13644369	17	33892241		N_Shelf	0.07	5.23	2.21E-07	6.36E-02	3	cg09356524	20	45898379	*ZMYND8*	OpenSea	0.03	4.79	2.19E-06	5.72E-01
4	cg03067296	17	76274577	*AC087645.1*	OpenSea	-0.06	-5.08	4.95E-07	6.68E-02	4	cg20515884	16	33605762		OpenSea	0.06	4.78	2.64E-06	5.72E-01
5	cg16317961	18	32621748	*MAPRE2*	S_Shore	0.05	5.07	5.16E-07	6.68E-02	5	cg20446201	10	1164931		OpenSea	0.03	4.71	3.32E-06	5.74E-01
6	cg25649869	10	37625532	*RP11–20F24.4*	OpenSea	-0.07	-5.06	5.30E-07	6.68E-02	6	cg25327296	11	44927093	*TSPAN18*	OpenSea	-0.03	-4.59	5.73E-06	8.26E-01
7	cg23110600	14	62012729	*PRKCH*	OpenSea	-0.06	-5.06	5.41E-07	6.68E-02	7	cg20294320	6	29617586		N_Shore	-0.04	-4.47	9.53E-06	9.93E-01
8	cg05702540	22	36861786		OpenSea	0.05	4.92	1.11E-06	1.03E-01	8	cg22549504	19	17448937		Island	0.07	4.45	1.10E-05	9.93E-01
9	cg05324894	14	105850192		N_Shore	0.06	4.91	1.11E-06	1.03E-01	9	cg21501831	12	108383714		OpenSea	0.03	4.45	1.13E-05	9.93E-01
10	cg17411016	2	47100912		OpenSea	-0.03	-4.90	1.19E-06	1.03E-01	10	cg25058917	2	222440185	*EPHA4*	S_Shore	0.05	4.40	1.38E-05	9.93E-01
11	cg12048674	1	205649805	*SLC45A3*	Island	-0.08	-4.85	1.55E-06	1.15E-01	11	cg12423311	7	153752727	*AC006019.3*	S_Shelf	0.07	4.37	1.58E-05	9.93E-01
12	cg14147906	2	67058274		OpenSea	-0.07	-4.84	1.60E-06	1.15E-01	12	cg01794889	6	28890399	*TRIM27*	N_Shore	-0.02	-4.36	1.60E-05	9.93E-01
13	cg27407935	17	17723235	*SREBF1*	N_Shelf	-0.06	-4.80	1.96E-06	1.25E-01	13	cg11959697	1	38942644		S_Shore	0.03	4.32	2.03E-05	9.93E-01
14	cg03546163	6	35654363	*FKBP5*	N_Shore	-0.11	-4.79	2.06E-06	1.25E-01	14	cg10491542	8	145161323	*KIAA1875*	N_Shore	-0.04	-4.30	2.04E-05	9.93E-01
15	cg03804474	5	67760417		OpenSea	-0.05	-4.76	2.37E-06	1.25E-01	15	cg03312142	19	44462423	*ZNF221*	OpenSea	-0.03	-4.30	2.08E-05	9.93E-01
16	cg07906745	1	109639152	*TMEM167B*	N_Shelf	0.05	4.75	2.43E-06	1.25E-01	16	cg15725372	7	27162153	*HOXA-AS2*	Island	-0.03	-4.25	2.51E-05	9.93E-01
17	cg21737354	10	71239238		OpenSea	0.03	4.75	2.47E-06	1.25E-01	17	cg21123835	2	179623903	*TTN*	OpenSea	0.02	4.25	2.60E-05	9.93E-01
18	cg25255847	20	62360072	*ZGPAT*	OpenSea	-0.06	-4.71	3.01E-06	1.38E-01	18	cg15120633	11	62559345	*TMEM223*	Island	-0.06	-4.25	2.61E-05	9.93E-01
19	cg25929046	22	23409066		N_Shelf	-0.03	-4.71	3.02E-06	1.38E-01	19	cg09880955	14	29240904	*C14orf23*	N_Shelf	0.07	4.24	2.77E-05	9.93E-01
20	cg23449570	6	11462069	*RP11–716O23.1*	OpenSea	-0.07	-4.67	3.50E-06	1.51E-01	20	cg01586844	19	38494937	*SIPA1L3*	OpenSea	-0.03	-4.23	2.82E-05	9.93E-01

same subject between the active injecting and abstinence visits ($p = 0.03$). Our analyses showed active IDU is positively associated with biological age acceleration compared with abstinence IDU visits within the same subject during the past six months.

4. Discussion

We conducted a comprehensive EWAS on active IDU, the intensity and type of injection use among a cohort of current and former PWID in a long-standing cohort. We found epigenome-wide significant CpG sites associated with active injecting (cg10636246, *AIM2*) and injection intensity (cg05324894). By comparing the top 0.1% differentially methylated CpG sites across five EWAS, we found converging evidence for cg10636246 (*AIM2*), cg23110600 (*PRKCH*), cg03546163 (*FKBP5*), cg04590956 (*GMCL1*), cg16317961 (*MAPRE2*), which were among top ranked CpG list across each EWAS. We also identified that the genes nearest to the top ranked CpG sites were enriched in AIM2 inflammasome complex pathway, T cell migration, insulin signaling pathway and epinephrine synthesis by gene ontology enrichment analyses. There was also significant difference in DNA methylation age as calculated by PhenoAge (Levine et al., 2018) between the active injecting and abstinence visits within the same subject.

There are possible biological mechanisms to explain the association between the identified CpGs and injection drug use. The AIM2 inflammasome plays a known role in detecting foreign DNA and plays an important role in inflammatory immune responses (Lugrin and Martinon, 2018; Rathinam et al., 2010). Injection drug use is associated with HIV infection and hepatitis C infection (Conrad et al., 2015; Page et al., 2013; Zibbell et al., 2018, 2015), and the increased risk of infection during periods of active IDU is reflected by the association between cg10636246 (*AIM2*) and active injecting. Mutations in *PRKCH* are associated with cerebral infarction and rheumatoid arthritis (Kubo et al., 2007; Takata et al., 2007) and *PRKCH* may play a role in immune response. *FKBP5* plays a central role in moderating glucocorticoid receptor responsiveness in the HPA-axis and has been among the most widely reported examples of differentially methylated genes in the pathway of stress related psychiatric disorders (Mahon et al., 2013; Matosin et al., 2018; Zannas et al., 2016). Polymorphisms and DNA methylation *FKBP5* have been shown to be associated with heroin dependence (Levran et al., 2014), alcohol withdrawal (Huang et al., 2014), post-traumatic stress disorder and depression (Binder et al., 2008; Klengel et al., 2013; Klinger-König et al., 2019; Matosin et al., 2018; Zannas et al., 2016). It is possible that differential *FKBP5* methylation is reflective of a chronic stress response associated with periods of active IDU. CpG sites associated with cocaine injection are enriched in insulin regulation and epinephrine synthesis pathways. Previous studies have shown that after intravenous cocaine administration there is a decrease in insulin (Bouhlal et al., 2017). The finding of enrichment in CpG associations with cocaine injection in epinephrine metabolic and biosynthetic process pathways is consistent with the known mechanism of action of cocaine – blocking norepinephrine reuptake, leading to an increase in norepinephrine and epinephrine concentrations. This is further supported by findings of an increase in plasma epinephrine after cocaine administration (Sofuoglu et al., 2001) and may elucidate the mechanism for the psychiatric sequelae of chronic cocaine use (Kosten et al., 1987; Lake et al., 1982; Ressler and Nemeroff, 1999).

Our analyses provided several CpG sites and genomic regions of interest, and, after replication, can be further explored by functional studies. However, compared to the previous EWAS on lifetime injection drug use and hepatitis C (Zhang et al., 2017), there was no overlap between the identified CpG sites and genes. Since we utilized a longitudinal design comparing subjects during periods of active injecting and abstinence, our focus was on the within-subject effect of active IDU on DNA methylation profiles; there might be innate differences in underlying biological pathways with the previous study comparing lifetime

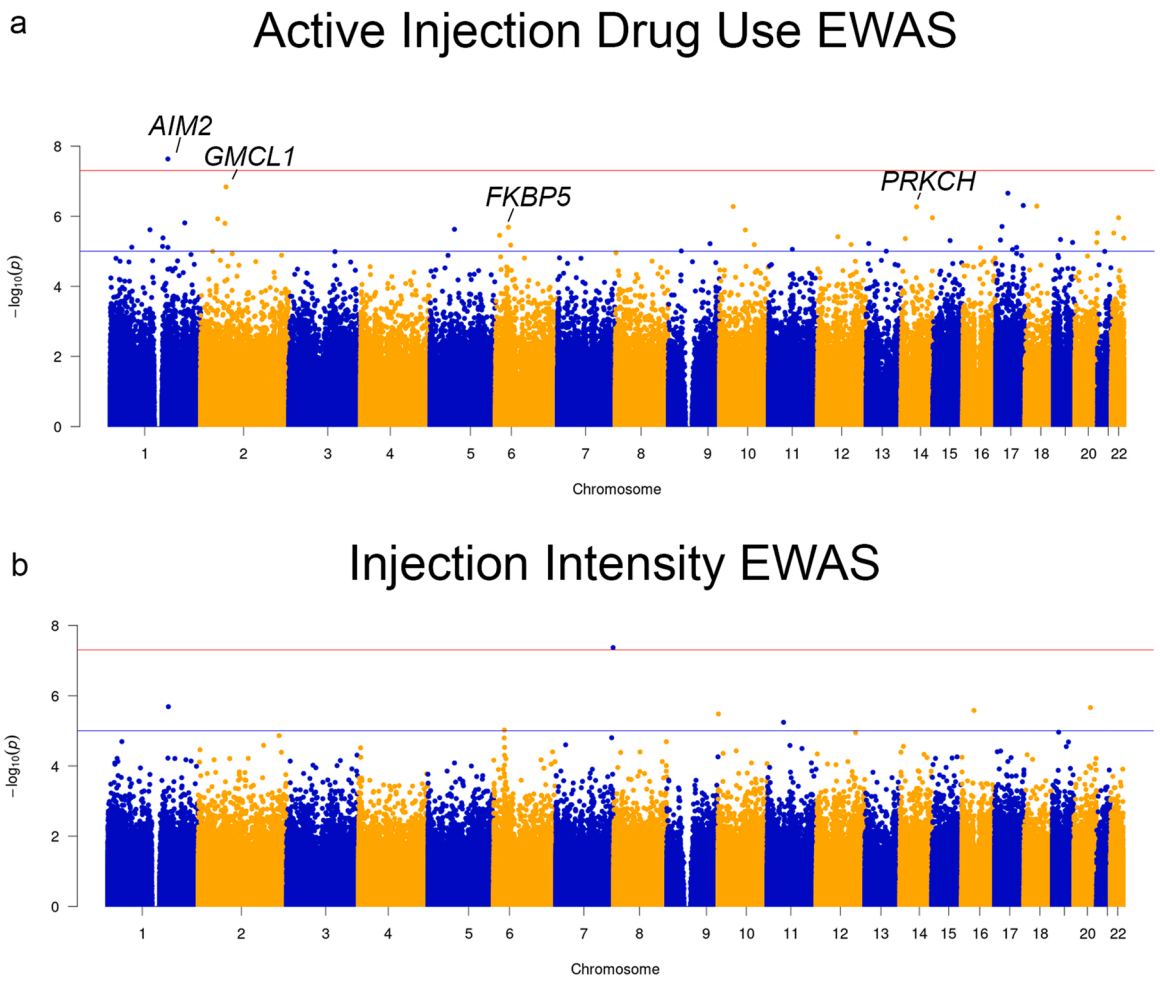

Fig. 1. Manhattan plots showing the epigenome-wide association study (EWAS) on active injecting (a) and injection intensity (b) in the past six months.

persons who inject drugs and those who never inject drugs. Moreover, there was no overlap between top ranked differentially methylated CpG sites between our heroin injection use EWAS and another recent EWAS on opioid dependence in European-American women (Montalvo-Ortiz et al., 2019). Our samples were predominately from African American participants that were 31.6% female and 68.4% male, which is a very different sample composition to the opioid dependence EWAS. Our study focused on any use of heroin injection in the past six months, whereas Montalvo-ortiz et al. compared opioid dependent to opioid-exposed controls. Despite the differences with previously published EWAS, we believe that with more data on DNA methylation among PWID, meta-analysis can be used to aggregate epigenetics findings across studies with similar IDU measures in this field and yield more reliable results.

We also found that there was significant biological age acceleration between active injecting and abstinence visits within the same subject. This finding highlighted the changes of DNA methylation age across time and its utility to evaluate temporal environmental exposure changes in longitudinal studies. Montalvo-ortiz et al. found there was no DNA methylation age difference between opioid dependence subjects and opioid-exposed controls (Montalvo-Ortiz et al., 2019). The results might not be directly comparable since we utilized a longitudinal study design to measure the DNA methylation age (PhenoAge) (Levine et al., 2018) difference within the same subject comparing periods of active and abstinence heroin injection. This finding is of particular interest because of the increased mortality rate among PWID. Much of this increased mortality is due to IDU-related infection and overdose (Kohli et al., 2005), but work in the ALIVE cohort has demonstrated that frailty independently contributes to mortality risk among PWID (Piggott et al., 2013). This and future work will further our understanding of the biological mechanisms of increased mortality risk among PWID, independent of other common causes.

There is a general lack of knowledge on how IDU affects DNA methylation in humans. Evidence on the effect of heroin and cocaine use in human and mouse models has focused on either DNA methylation on specific genes like *OPRM1*, or global methylation or demethylation (Chao et al., 2014; Nielsen et al., 2009). The development of methylation profiling microarray technology allowed us to measure hundreds of thousands of CpG sites at lower cost and conduct EWAS. EWAS on smoking behavior and alcohol consumption have revealed the utility of epigenetic sites as biomarkers for individual smoking, maternal smoking, and alcohol consumption (Joubert et al., 2012; Ladd-Acosta et al., 2016; Liu et al., 2016; Tsaprouni et al., 2014). It is highly possible that there exist epigenetic markers associated with injection drug use of heroin or cocaine, which will require larger samples to reproducibly detect due to small effect sizes. While we lack general knowledge, our study gives us a glimpse into how dynamic the DNA methylation change can be across periods of active injecting and abstinence.

The strength of our study is that we took advantage of the longitudinal study design and provided insights on the within-person DNA methylation as well as DNA methylation age change between active and abstinence injection periods. The DNA methylation profile was reflective of the past six-month active injection status and our study focused on a more appropriate time window of biological relevance. Importantly, all subjects in our sample were chronic PWID, most with many years of drug use. It is likely that long-term drug use has a lasting impact

Table 3
Epigenome-wide association analysis of heroin injection use and cocaine injection use during the prior six months.

Heroin injection use										Cocaine injection use									
Rank	CpG	Chr	Position	Gene	Relation to Island	Estimate	t value	p value	FDR	Rank	CpG	Chr	Position	Gene	Relation to Island	Estimate	t value	p value	FDR
1	cg10636246	1	159046973	*AIM2*	OpenSea	-0.15	-5.23	2.29E-07	1.10E-01	1	cg14347670	6	41908995	*CCND3*	Island	-0.14	-5.28	1.87E-07	1.60E-01
2	cg23110600	14	62012729	*PRKCH*	OpenSea	-0.07	-5.16	3.13E-07	1.10E-01	2	cg24814826	1	227579822	*CTD-2090I13.1*	OpenSea	0.10	5.09	4.83E-07	1.60E-01
3	cg27407935	17	17723235	*SREBF1*	N_Shelf	-0.07	-5.13	3.80E-07	1.10E-01	3	cg26520396	13	106954459		OpenSea	-0.07	-5.05	5.57E-07	1.60E-01
4	cg03546163	6	35654363	*FKBP5*	N_Shore	-0.13	-4.86	1.45E-06	1.80E-01	4	cg16317961	18	32621748	*MAPRE2*	S_Shore	0.06	4.98	8.76E-07	1.61E-01
5	cg16583817	10	104005140	*GBF1*	Island	0.09	4.75	2.40E-06	1.80E-01	5	cg05451974	20	43991922	*SYS1*	Island	0.08	4.95	9.30E-07	1.61E-01
6	cg12864116	19	44191141		OpenSea	-0.04	-4.76	2.41E-06	1.80E-01	6	cg01525244	22	39548611	*CBX7*	N_Shore	0.05	4.92	1.25E-06	1.80E-01
7	cg07195224	1	159047034	*AIM2*	OpenSea	-0.11	-4.75	2.56E-06	1.80E-01	7	cg22645881	17	37824988	*PNMT*	Island	0.05	4.87	1.53E-06	1.89E-01
8	cg03804474	5	67760417		OpenSea	-0.06	-4.74	2.70E-06	1.80E-01	8	cg21737354	10	71239238		OpenSea	0.05	4.78	2.25E-06	2.34E-01
9	cg03067296	17	76274577	*AC087645.1*	OpenSea	-0.06	-4.74	2.72E-06	1.80E-01	9	cg23005227	3	50645426		N_Shelf	0.06	4.77	2.43E-06	2.34E-01
10	cg05702540	22	36861786		OpenSea	0.05	4.73	2.84E-06	1.80E-01	10	cg02595922	7	1014709		Island	0.06	4.68	3.50E-06	2.79E-01
11	cg12048674	1	205649805	*SLC45A3*	Island	-0.08	-4.72	2.88E-06	1.80E-01	11	cg00854963	X	70364566	*NLGN3*	OpenSea	0.08	4.69	3.55E-06	2.79E-01
12	cg25242306	13	74667131	*KLF12*	OpenSea	0.06	4.71	2.99E-06	1.80E-01	12	cg17286253	1	185703489	*HMCN1*	Island	-0.07	-4.63	4.52E-06	2.93E-01
13	ch 0.4.75060805 F	4	74841941		OpenSea	0.10	4.71	3.03E-06	1.80E-01	13	ch 0.10.2633792 F	10	123987885		OpenSea	0.11	4.61	4.69E-06	2.93E-01
14	cg26313514	17	7338701	*TMEM102*	N_Shore	-0.05	-4.70	3.10E-06	1.80E-01	14	cg15886862	X	12026390		OpenSea	-0.05	-4.63	4.74E-06	2.93E-01
15	cg10695583	17	48625306	*SPATA20*	S_Shore	-0.03	-4.71	3.11E-06	1.80E-01	15	cg12048674	1	205649805	*SLC45A3*	Island	-0.09	-4.59	5.22E-06	3.01E-01
16	cg24938072	17	13460471		OpenSea	-0.05	-4.66	3.77E-06	2.04E-01	16	cg11203815	3	128434169	*SNORA24*	OpenSea	0.09	4.55	6.48E-06	3.44E-01
17	cg25076285	18	43948046	*RNF165*	OpenSea	-0.04	-4.63	4.39E-06	2.07E-01	17	cg12996903	3	50275575	*GNAI2*	Island	-0.07	-4.52	7.49E-06	3.44E-01
18	cg13644369	17	33892241		N_Shelf	0.07	4.62	4.67E-06	2.07E-01	18	cg23110600	14	62012729	*PRKCH*	OpenSea	-0.07	-4.51	7.56E-06	3.44E-01
19	cg10126903	16	29675214		N_Shore	0.07	4.61	4.89E-06	2.07E-01	19	cg21652108	12	12944951	*RP11–59H1.3*	OpenSea	0.08	4.53	7.57E-06	3.44E-01
20	cg05531044	3	132441182	*NPHP3*	Island	-0.07	-4.60	4.96E-06	2.07E-01	20	cg25255847	20	62360072	*ZGPAT*	OpenSea	-0.08	-4.50	8.41E-06	3.63E-01

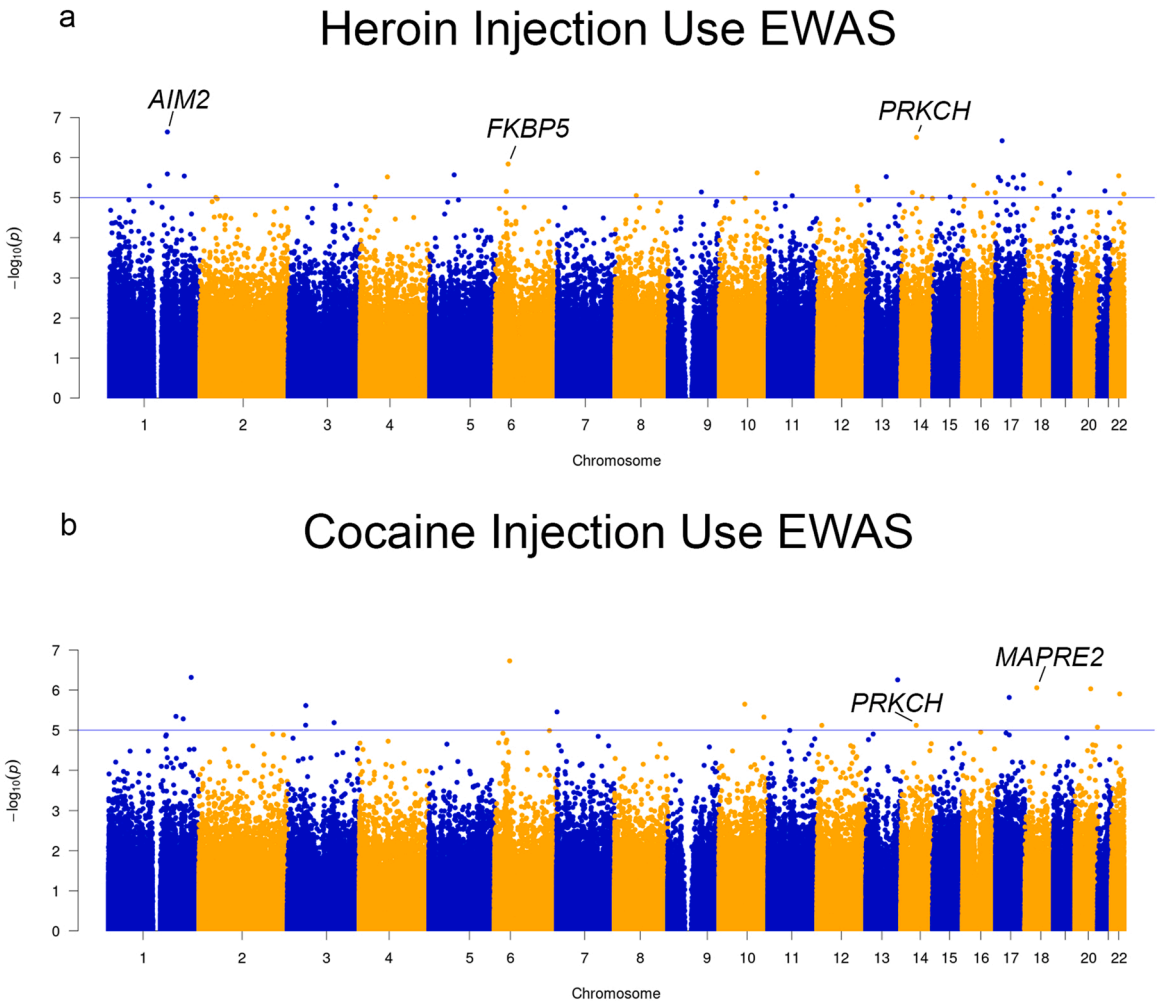

Fig. 2. Manhattan plots showing the epigenome-wide association study (EWAS) on past six month heroin injection use (a) and cocaine injection use (b).

on the epigenome. In an attempt to account for this, we included multiple visits per subject, some with visits abstinence visits preceding active use visits and some with use visits preceding abstinence visits. However, future research should focus on disentangling the epigenetic signatures representing ever versus never use, use versus abstinence among chronic users and the quantitative impact of long-term use, since

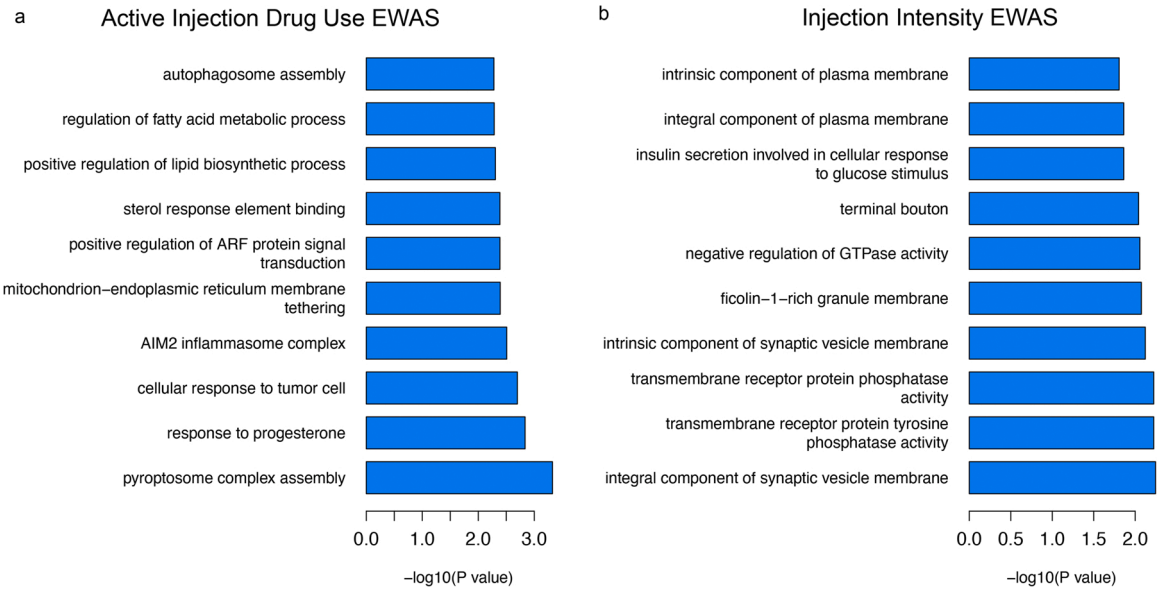

Fig. 3. Gene ontology enrichment analysis among top epigenome-wide association study (EWAS) hits on the past six months of any injection drug use (a) and frequency of injection drug use (b). Nominal p-values are shown.

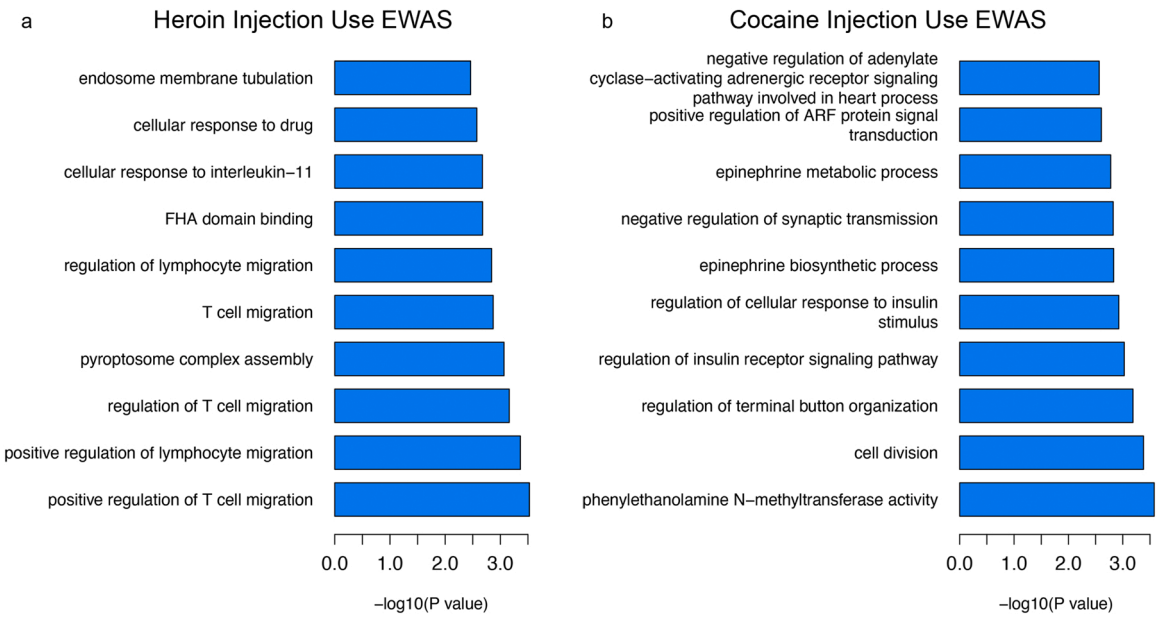

Fig. 4. Gene ontology enrichment analysis among top epigenome-wide association study (EWAS) hits on the past six months for heroin injection use (a) and cocaine injection use (b). Nominal p-values are shown.

each of these represent different stages in the trajectory of substance use, dependence and cessation. Additionally, our study sample consists mainly of African Americans and we contributed more genetics data in this population that is underrepresented in the literature.

The primary limitation of our study is that the active injection status is based on self-report and may subject to recall bias. Additionally, our study only focused on injection drug use and did not account for other routes of drug administration. There is also sample overlap between heroin and cocaine injection drug users, making it difficult to disentangle specific effects.

In conclusion, our study identified several CpG sites associated with active injection drug use, injection intensity, and heroin injection use during the past six months. We found that injection drug use significantly accelerated biological age during periods of active injecting compared with abstinence visits within the same subject. We showed the utility of longitudinal study design in EWAS and its ability to inform within-subject changes in epigenetic profiles induced by active injection drug use.

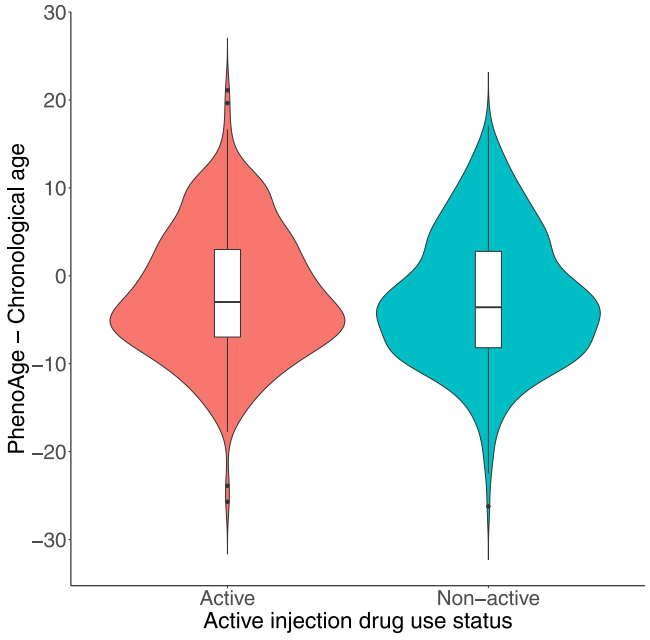

Fig. 5. Biological age acceleration based on the difference of DNA methylation age (PhenoAge) and chronological age by past six month injection drug use status.

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CRediT authorship contribution statement

CS analyzed data and wrote the manuscript. BM designed the methylation study. AJ and HJ provided methodological guidance. SS, JS, KB, DS helped prepare manuscript. JA was responsible of data management and extraction. GK and SM were responsible for participant recruitment and design of the ALIVE study. All authors contributed to manuscript preparation.

Data Availability

The data that support the findings of this study will be openly available in dbGaP: Accession number: phs001494.

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Conflict of Interest

SM has the following disclosures: speaker fees from Gilead Sciences. PI for research grants - funds paid to Johns Hopkins University: AbbVie, Assembly Bio, Gilead, Proteus Digital Health. Scientific advisor/

Consultant: The terms of these arrangements are being managed by the Johns Hopkins University in accordance with its conflict of interest policies: AbbVie, Arbutus, Gilead. Other authors declare that there is no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.drugalcdep.2022.109431.

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