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Global DNA methylation levels in white blood cells of patients with chronic heroin use disorder. A prospective study

Domniki Fragou [a], Mu-Rong Chao [b], Chiung-Wen Hu [c], Kakia Nikolaou [d], Leda Kovatsi [a,*]

[a] Laboratory of Forensic Medicine and Toxicology, Department of Medicine, Aristotle University of Thessaloniki, Greece
[b] Department of Occupational Safety and Health, Chung Shan Medical University, Taichung 402, Taiwan
[c] Department of Public Health, Chung Shan Medical University, Taichung 402, Taiwan
[d] Addiction Department IANOS, General Hospital of Thessaloniki 'G. Papanikolaou'-Psychiatric Hospital of Thessaloniki, Thessaloniki, Greece

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ABSTRACT

Background: Increasing scientific evidence shows the significant role of epigenetic mechanisms in drug use disorder, abstinence and relapse. Studies on human subjects are limited compared to those on animals, for various reasons such as poly-substance abuse, high drop-out rate and technical difficulties.
Objectives: Our goal was to evaluate whether a monitored abstinence period of 21 days could induce changes in global DNA methylation in chronic heroin users.
Method: In the current study, we present data on global DNA methylation on a set of 18 male patients with chronic heroin use disorder, carefully selected based on inclusion and exclusion criteria, who were hospitalized and closely monitored during a 21-day detoxification program, one of the few where no opioid agonist is administered. The participants were sampled twice, once upon enrolment to the program and once upon completion.
Results: According to our results, no difference in global DNA methylation was detected between samples collected upon enrolment and samples collected upon completion of the program.
Conclusion: The findings of this study do not rule out the possibility that the 21-day abstinence period was not long enough to observe changes in global DNA methylation, or that abstinence induced site-specific methylation changes (but not global changes), that certainly merit further evaluation.

1. Introduction[1]

Epigenetic changes refer to inherited or acquired changes of the epigenome which do not alter the DNA sequence and which can be reversed. These include histone modifications such as methylation, acetylation, phosphorylation, ubiquitination and DNA methylation or acetylation. DNA methylation, in particular, involves the addition of a methyl group in the 5′ carbon of the cytosine ring, which leads to the formation of 5′ methylcytosine, which is known as the "fifth base" of DNA. DNA methylation is a state which exists naturally and can be passed on from parents to offspring. It is subject to changes throughout a person's life course, by various factors, such as environmental stimuli. Such environmental factors include exposure to toxicants and environmental pollutants, smoking and dietary habits, as well as drug use. Furthermore, DNA methylation can play a mechanistic role in the onset and progression of diseases such as cancer, heart disease, fetal syndromes and neurodegenerative disorders [1].

DNA methylation occurs mainly in genomic regions which are rich in CpG sites, called CpG islands. An increase in DNA methylation will cause a more condensed chromatin structure, associated with gene silencing, whereas a decrease in DNA methylation will have the opposite effect, i.e. a more relaxed chromatin structure and active gene transcription. Demethylation can be compensated by the addition of methyl groups facilitated by certain enzymes called DNA methyltransferases. On the other hand, 5′-methylcytosine (5-mC) can be converted to 5′-hydroxymethylcytosine (5-hmC) by the ten-eleven translocation proteins. It is subsequently converted to 5′-hydroxymethyluracil by deaminases in order to be repaired to cytosine by the base excision repair pathway

* Corresponding author at: Laboratory of Forensic Medicine and Toxicology, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece.
E-mail addresses: domnikif@hotmail.com (D. Fragou), chaomurong@gmail.com (M.-R. Chao), kakianik@gmail.com (K. Nikolaou), kovatsi@auth.gr (L. Kovatsi).

[1] 5-mC: 5'-methylcytosine, 5-hmC: 5'-hydroxymethylcytosine, d3−5-mdC: d3−5-methyl-2'-deoxycytidine, d3−5-hmdC: d3−5-hydroxymethyl-2'-deoxycytidine, NAS: Neonatal Abstinence Syndrome

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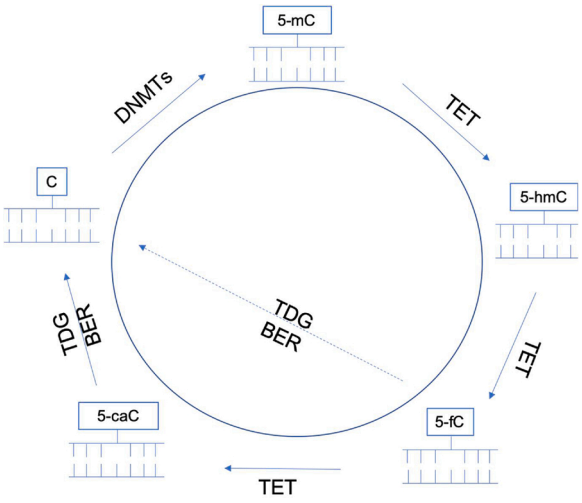

Fig. 1. DNA methylation repair pathway.
5-mC is converted to 5-hmC→5-fC→5-caC by TET proteins and 5-caC is converted to C by TDG, BER. (5-fC: 5-formylcytosine, 5-caC: 5-carboxylcytosine, TET: ten-eleven translocation, TDG: thymine DNA glycosylase, BER: base excision repair)

(Fig. 1) [2]. Therefore, whatever the exogenous effect on DNA methylation status, it can be reversed either by natural pathways, or by means of methylating or demethylating agents that mimic the natural processes.

In recent years, DNA methylation has been associated with opiate use and/or dependence [3]. Both acute and chronic morphine exposure have been associated with brain region-specific changes in 5-mC and 5-hmC levels [4]. Moreover, gene specific hypo-methylation has been reported to coincide with heroin use disorder in animal models and has been associated with the treatment and prevention of opioid use [5]. An epigenome-wide association study that was conducted in 220 European-American women revealed three differentially methylated regions associated with genes PARG, RERE, and CFAP77 [6]. Differentially methylated CpG sites were also found in post-mortem samples obtained from heroin users - neuronal nuclei from the orbitofrontal cortex [7].

Another gene implicated in opioid use disorder is the OPRM1 gene. Increased methylation in certain CpG sites of the gene has been reported in former heroin addicted individuals in the Chinese Han population [8] and in the lymphocytes of former heroin addicted individuals, currently on methadone maintenance [9]. In the Xu et al. study, only one CpG site was significantly hypomethylated in former addicted individuals when compared to controls [8]. Seven CpG sites of the gene were found to be hypermethylated in blood from opioid addicts and one site in the sperm of the addicts suggesting possible mechanisms of dependence phenotype heritability [10]. Furthermore, diacetylmorphine-assisted treatment of heroin addiction caused hypermethylation of the pro- opiomelanocortin gene in blood samples [11]. In a recent study on oxytocin, a neuropeptide which is gaining increasing interest because there is data supporting that it could reduce the use potential of drugs, oxytocin was found to inhibit the global 5-mC changes induced by oxycodone conditioned place preference [12]. Furthermore, it was recently shown that ten-eleven translocation (Tet) enzymes, which are responsible for DNA demethylation, may play a role in the prevention of opioid use disorder, since they can modify the morphine-seeking behavior [13].

One of the biggest challenges in drug use disorder is relapse, even after long periods of abstinence. There is mounting scientific evidence that epigenetic factors are implicated in the persistence of drug use disorder [14]. Infants that develop Neonatal Abstinence Syndrome (NAS) after in-utero opioid exposure, present with withdrawal symptoms 2–3 days after birth and are treated with opioid substitutes such as methadone, morphine or buprenorphine [15]. In a recent article on the epigenetics of NAS, the authors conclude that the epigenetic profile may predict NAS severity, may explain the individualized response to pharmacotherapy and may help towards the development of tailored and personalized treatments [16], which constitute a big challenge for the transformation of healthcare services [17], along with the field of miRNA studies [18,19–25].

There is currently limited scientific information on how long it takes for DNA methylation to be restored, once the etiological factor has been removed. In a recent review by [26], the authors report that most epigenetic changes remain persistent from 2 to 30 days after the last drug exposure across various drug classes [26], but such wide variation requires more research on this issue.

In the present study, we have prospectively studied (at two different time points) a cohort of patients chronically addicted to heroin. Blood samples were collected upon their enrolment in a detoxification program, as well as following 21 days of abstinence. We evaluated 21 days of abstinence as an initial trial period because this is the period during which the subjects are closely monitored in the residential facility and abstinence can be guaranteed. This time-frame is reasonable according to the available scientific information. The samples were analysed for 5-mC global levels in order to evaluate changes in the global DNA methylation status after completion of the 21-day “dry” program. Furthermore, samples were analysed for 5-hmC in order to evaluate whether repair mechanisms that convert 5-mC to 5-hmC are triggered.

2. Materials & methods

2.1. Participants

72 individuals chronically addicted to heroin, entered voluntarily a 21-day detoxification program. Only 36 of them managed to complete the program. Out of these, only 18 fulfilled the study inclusion criteria (white Caucasian males, with heroin as the only drug of abuse, with a limited alcohol consumption, with no history of autoimmune diseases, diabetes, hypertension, Crohns’ disease and cardiovascular-, renal- or pulmonary-related disorders), further limiting the number of participants finally included in the study (Fig. 2). Individuals who reported intake of drugs other than heroin (including illicit fentanyl and prescription opioids), or an alcohol consumption >300 mL/wk were excluded from the study because we wanted to focus on single opioid users in order to ensure homogeneity and comparability of the participants. Additionally, those with a history of autoimmune diseases, diabetes, hypertension, Crohns’ disease and cardiovascular-, renal- or pulmonary-related disorders were also excluded from the study. The participants were all white Caucasian males, with a mean age of 33 ± 6 years (ranging from 25 to 48 years). Out of the 18 participants, thirteen were positive for anti−HCV antibodies. Opioid dependence was diagnosed in year 2009 in all volunteers according to the criteria set by the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) [27].

All participants enrolled voluntarily in the 21-day detoxification program which is operated by the “Ianos” Addiction Department, a residential facility of the Psychiatric Hospital of Thessaloniki, Greece. This is a “dry” detoxification program i.e. no opioid agonist is administered to participants during the 21-day program. During their stay in the residential facility of the Psychiatric Hospital of Thessaloniki, abstinence was monitored with urine drug screening without advance notice. Users found positive, were expelled from the program.

Upon entering the program, all participants were interviewed according to the Addiction Severity Index questionnaire (ASI) [28]. Individuals included in the study tested positive for heroin in the urine drug test and all had a prior continuous use of 6 months or more. The average length of heroin use was 13.3 years and the average daily dose was 1.25 g of street heroin (averagely 17 % pure heroin). Institutional Review Board (IRB) approval was obtained from the Bioethics Committee of the Psychiatric Hospital of Thessaloniki, Greece.

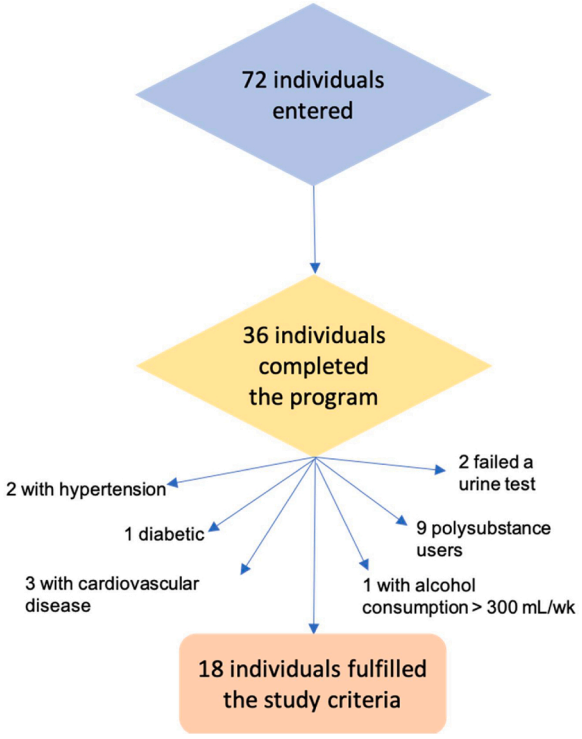

Fig. 2. Flow chart explaining the gradual limitation of the subjects included in the study.

Blood samples were drawn at two time points: upon entering the program and upon completion of the 21-day detoxification period. Blood collection was carried out in the morning, after a 12 -h fasting period. White blood cells were isolated from blood by centrifugation and stored at -35 °C until analysis.

All participants signed an informed consent form. The study was conducted in accordance to the Declaration of Helsinki [29].

2.2. Method

DNA was extracted from white blood cells using the QIAamp DNA mini kit, following the manufacturer's instructions.

The isolated DNA samples were enzymatically digested and analysed as described by Hu et al. [30] with some modifications. Briefly, 10 μL of 0.1 U/μL nuclease P1 (in 300 mM sodium acetate and 1 mM ZnSO4, pH 5.3) were added to the DNA samples, followed by incubation at 37 °C for 2 h. Thereafter, 10 μL of 10X alkaline phosphatase buffer (500 mM Tris/HCl, pH 8.5, 1 mM EDTA) together with 4 μL of alkaline phosphatase (1 U/μL) were added and the incubation was continued at 37 °C for 2 h. The crude DNA hydrolysates were then neutralized with 10 μL of 0.5 N HCl, spiked with 50 ng of d3−5-methyl-2′-deoxycytidine (d3−5-mdC) and 1 ng of d3−5-hydroxymethyl-2′-deoxycytidine (d3−5-hmdC) as internal standards, followed by online solid phase extraction (online SPE) LC–MS/MS analysis.

The online SPE instrumentation consisted of a 10-port two-position switching valve (Valco, Instruments) and a C18 trap column (33 × 2.1 mm i.d., 5 μm, ODS-3, Inertsil) and was controlled by PE-SCIEX control software (Analyst, Applied Biosystems). After the online sample purification procedure, the sample was directly transferred to a C18 column (150 × 2.1 mm i.d., 5 μm, ODS-3, Inertsil) to separate the analytes. The total run time was 15 min. The target analyte was quantified by an API 4000 QTrap hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) equipped with a TurboIon-Spray source. The resolution was set to a peak width (FWHM) of 0.7 Th for both quadrupole Q1 and Q3. Detection was performed in the positive ion multiple reaction monitoring (MRM) mode for simultaneous quantitation of 5-mdC and 5-hmdC.

2.3. Statistical analysis

All statistical analyses were carried out using the SPSS software v. 18. Continuous variables were expressed as percentage of total cytosine content using the equation:

$$\%5-\mathrm{mdC}=\frac{5-mdC(nmol)}{dC(nmol)+5-mdC\ (nmol)+5-hmdC(nmol)}x100$$

Continuous variables were assessed with the Kolmogorov-Smirnov test for normality. Since the distribution was not normal the non-parametric Wilcoxon Signed-Rank test was used to compare the global DNA methylation levels of the same individuals between two different time points, with a confidence interval of 95 %. $p < 0.05$ was considered to be statistically significant.

3. Results

Representative online SPE LC–MS/MS chromatograms of standards in the neat solution and of DNA isolated from white blood cells are shown in Fig. 3 and Supplementary Fig. S1, respectively.

In this study, 5-hmdC was not detectable in all DNA samples, probably because an insufficient DNA amount (< 1 μg) was obtained from some samples. 2′-Deoxycytidine (dC) was measured using a previously reported LC–MS/MS method following enzymatic hydrolysis [31]. The levels of 5-mdC present in the DNA sample were expressed as a percentage of total cytosine content (dC, 5-mdC and 5-hmdC) using the equation described in the materials section and are shown in Table 1.

Statistical analysis of the raw data revealed that there is no significant difference ($p = 0.647$) in the levels of global DNA methylation between the two different time points, i.e. upon entering and after completion of the 21-day "dry" detoxification program.

4. Discussion

In the work currently presented, we evaluated the effect of abstinence on global DNA methylation levels in patients with chronic heroin use disorder. Participants were sampled twice: upon enrolling in a 21-day detoxification program and upon completion of the program. To the best of our knowledge, this is the first study on such a unique set of individuals chronically addicted to heroin, who were hospitalized and closely monitored during a 21-day "dry" detoxification program (no opioid agonist was administered during this program). Furthermore, it is the first time that such a population is studied prospectively, enabling the comparison between global DNA methylation levels under opioid dependence and those following 21 days of abstinence.

According to our results, the 21-day abstinence period did not affect the total genomic methylation levels. Nevertheless, this finding does not imply that this abstinence period did not trigger epigenetic changes, at specific sites. It could be that transcription start sites and CG islands were hypermethylated and flanking regions were hypomethylated, or vice versa, and thus these two effects cancelled each other out. As Imperio et al. [5] showed, heroin administration to rats revealed no differences in total genomic methylation, but induced site-specific hypo-methylation in EGR1 and EGR2. Moreover, differential methylation was observed in six nuclear-encoded genes in different rat brain regions, following acute and chronic morphine administration [4]. Furthermore, three differentially methylated CpG sites that correspond to the *PARG*, *RERE*, and *CFAP77* genes were identified in a genome wide analysis of whole blood samples from opioid exposed women (Montalvo-Ortiz et al., 2018). These studies indicate that although total genomic DNA methylation might be stable, site- specific changes in DNA methylation may occur.

The findings of this study are similar to the findings of previous

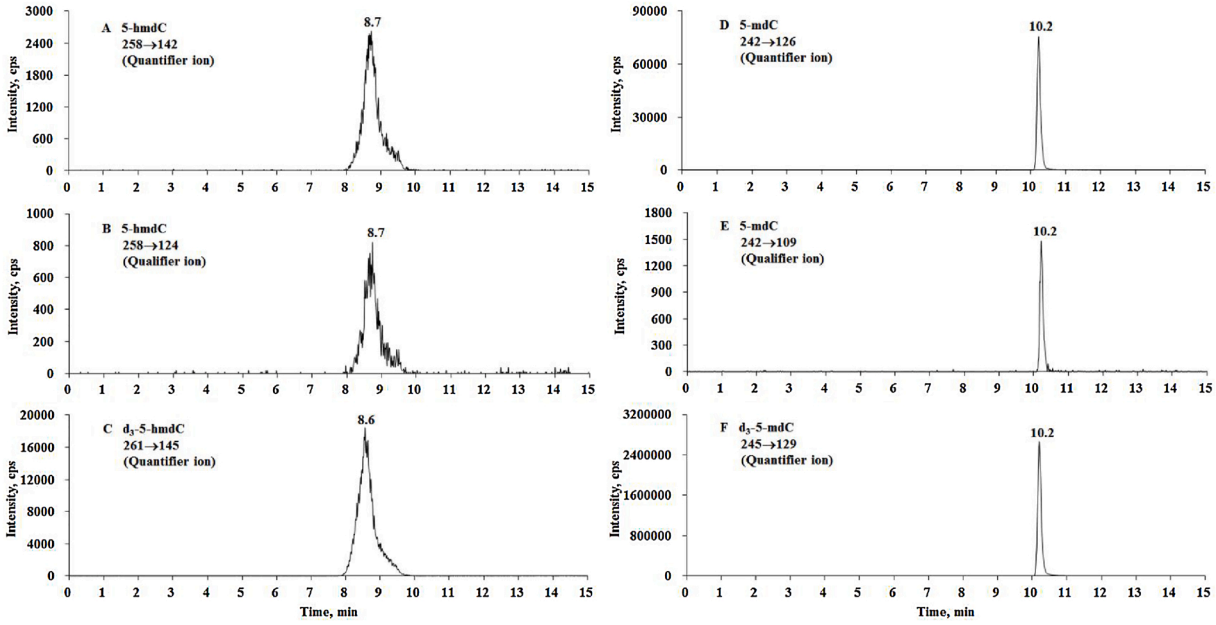

Fig. 3. 5-hmdC (1.25 ng/mL) and 5-mdC (15.6 ng/mL) in the neat solution. Chromatograms generated by LC–MS/MS coupled with online SPE. MRM transitions of (A) *m/z* 258→142 and (B) *m/z* 258→124 for 5-hmdC, and (C) *m/z* 261→145 for d3-5-hmdC; (D) *m/z* 242→126 and (E) *m/z* 242→109 for 5-mdC, and (F) *m/z* 245→129 for d3-5-mdC. cps, counts per second.

Table 1
dC and 5-mdC amounts (nmol) and global methylation levels in DNA isolated from white blood cells of patients with chronic heroin use disorder upon entering (IN) and after completion of the 21-day "dry" detoxification program (OUT).

Sample No	IN			OUT		
	dC (nmol)	5-mdC (nmol)	Methylation level (%)	dC (nmol)	5-mdC (nmol)	Methylation level (%)
1	0.00415	0.000022	0.52	0.00786	0.000243	3.00
2	0.00810	0.000276	3.30	0.00581	0.000135	2.27
3	0.00727	0.000191	2.56	0.01343	0.000425	3.07
4	0.00885	0.000291	3.18	0.00582	0.000161	2.69
5	0.00793	0.000281	3.43	0.00465	0.000063	1.33
6	0.00614	0.000151	2.40	0.00603	0.000156	2.51
7	0.00673	0.000316	4.49	0.00709	0.000204	2.79
8	0.00351	0.000025	0.71	0.00516	0.000088	1.67
9	0.00593	0.000148	2.43	0.00400	0.000056	1.39
10	0.01062	0.000347	3.16	0.00797	0.000256	3.12
11	0.00528	0.000109	2.02	0.00618	0.000213	3.32
12	0.00896	0.000321	3.46	0.00518	0.000101	1.91
13	0.00501	0.000460	8.41	0.00474	0.000098	2.02
14	0.00482	0.000066	1.35	0.00775	0.000244	3.06
15	0.00762	0.000209	2.67	0.01839	0.000584	3.08
16	0.01004	0.000330	3.18	0.00704	0.000185	2.56
17	0.00667	0.000178	2.60	0.01136	0.000381	3.25
18	0.00842	0.000276	3.17	0.01684	0.000555	3.19

studies we have conducted. When we investigated the global DNA methylation levels in the brain and liver of mice which were submitted to a heroin administration program mimicking heroin use disorder, and when we compared the treated mice to the saline treated control group, no difference in global DNA methylation was found [2,32].

Overall, it appears that global DNA methylation may not be an appropriate biomarker in the case of opioid use since differentially modified sites on the genome may cancel each other out, leading to an overall unchanged global methylation status. Site-specific methylation analysis could be more appropriate for determining the underlying mechanisms of opioid use disorder and detoxification processes and for developing more personalized and effective detoxification treatments.

The initial goal of this study was to measure both 5-mdC and 5-hmdC. Unfortunately, 5-hmdC could not be quantified in all samples. Therefore, the unobserved difference in the 5-mdC levels between the two time points could be interpreted in three ways: a) the detoxification process did not alter the global DNA methylation levels, b) the DNA methylation levels were indeed altered but due to the onset of the repair mechanisms this effect could not be identified by measuring 5-mdC alone, or c) the 21-days were not enough to notice any observable change in the global DNA methylation status. It is true that had we been able to measure the 5-hmdC levels, we would have a more precise explanation for our findings. The measurement of 5-hmdC is essential for future research since it will provide valuable information on the underlying mechanisms that are responsible for changes in the global DNA methylation status. Moreover, in future studies a longer abstinence period, greater than 21 days, might be a better approach to ensure that non-observable changes are not due to an insufficient abstinence period.

To the best of our knowledge, the work currently presented is novel in terms of study design: individuals with chronic heroin use disorder, carefully selected based on inclusion and exclusion criteria, who were hospitalized and closely monitored during a 21-day abstinence period,

with no opioid agonist administered during this time. This program is one of the few remaining in Greece where no opioid agonist is administered during detoxification. The drop-out rate is approximately 50 %. This explains that fact that the number of individuals initially enrolled in the study was much larger. Furthermore, the strict inclusion criteria in this study, as well as various technical problems (such as clogged veins which made blood collection impossible) further decreased the number of participants finally included in the study. All the above reasons justify the relatively small number of participants, which is a limitation of the work currently presented.

Another study is currently underway, on users who successfully complete the 21-day program and continue with another, outpatient, program in a therapeutic community. This will enable long-term follow up, but will require a long period of time for sample collection since the users may drop-out at several stages of the therapeutic process.

5. Conclusions

In conclusion, we currently present data on global DNA methylation in a unique set of patients with chronic heroin use disorder, who were prospectively studied during a 21-day detoxification program where no opioid agonist was administered. According to our results, no difference in global DNA methylation was detected between samples collected upon enrollment to the program and samples collected upon completion of the program. This finding does not rule out the possibility that the 21-day abstinence period induced site-specific methylation changes, that certainly merit further evaluation. Furthermore, one cannot rule out the possibility that 21 days are not enough for changes to occur. If this is the case, our findings may be useful in understanding the timelines for changes that might occur.

Contributors

Dr. D Fragou was responsible for experimental design, statistical analysis and manuscript preparation. Dr. MR Chao and Dr. CW Hu were responsible for development of analytical method and analysis of samples. Dr. K. Nikolaou was responsible for recruitment, sampling and follow-up of subjects. Dr. L Kovatsi developed the study concept, performed all regulatory work and finalized the manuscript. All authors have read and approved this manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.02.006.

References

[1] L. Kovatsi, D. Fragou, V. Samanidou, S. Njau, S. Kouidou, Drugs of abuse: epigenetic mechanisms in toxicity and addiction, Curr. Med. Chem. 18 (12) (2011) 1765–1774, https://doi.org/10.2174/092986711795496836.

[2] D. Fragou, P. Zanos, S. Kouidou, N. Samuel, K. Ian, A. Bailey, L. Kovatsi, Effect of chronic heroin and cocaine administration on global DNA methylation in brain and liver, Toxicol. Lett. 218 (3) (2013) 260–265, https://doi.org/10.1016/j.toxlet.2013.01.022.

[3] C.J. Browne, A. Godino, M. Salery, E.J. Nestler, Epigenetic mechanisms of opioid addiction, Biol. Psychiatry 87 (1) (2020) 22–33, https://doi.org/10.1016/j.biopsych.2019.06.027.

[4] T.M. Barrow, H.M. Byun, X. Li, C. Smart, Y.X. Wang, Y. Zhang, A.A. Baccarelli, L. Gup, The effect of morphine upon DNA methylation in ten regions of the rat brain, Epigenetics 12 (12) (2017) 1038–1047, https://doi.org/10.1080/15592294.2017.1398296.

[5] C.G. Imperio, A.J. McFalls, N. Hadad, L. Blanco-Berdugo, D.R. Masser, E. M. Colechio, A.A. Coffey, G.V. Bixler, D.R. Stanford, K.E. Vrana, P.S. Grigson, W. M. Freeman, Exposure to environmental enrichment attenuates addiction-like behavior and alters molecular effects of heroin self-administration in rats, Neuropharmacology 139 (2018) 26–40, https://doi.org/10.1016/j.neuropharm.2018.06.037.

[6] J.L. Montalvo-Ortiz, Z. Cheng, H.R. Kranzler, H. Zhang, J. Gelernter, Genomewide Study of Epigenetic Biomarkers of Opioid Dependence in European- American Women [published correction appears in Sci Rep. 2019 Dec ;9(1):18774], Sci. Rep. 9 (1) (2019) 4660, https://doi.org/10.1038/s41598-019-41110-7.

[7] A. Kozlenkov, A.E. Jaffe, A. Timashpolsky, P. Apontes, S. Rudchenko, M. Barbu, W. Byne, Y.L. Hurd, S. Horvath, S. Dracheva, DNA methylation profiling of human prefrontal cortex neurons in heroin users shows significant difference between genomic contexts of hyper- and hypomethylation and a younger epigenetic age, Genes 8 (6) (2017) 152, https://doi.org/10.3390/genes8060152.

[8] J. Xu, T. Wang, Z. Su, X. Zhou, Y. Xiang, L. He, C. Li, Z. Yang, X. Zhao, Opioid exposure is associated with aberrant DNA methylation of OPRM1 promoter region in a Chinese Han population, Biochem. Genet. 56 (5) (2018) 451–458, https://doi.org/10.1007/s10528-018-9852-y.

[9] D.A. Nielsen, V. Yuferov, S. Hamon, C. Jackson, A. Ho, J. Ott, M.J. Kreek, Increased OPRM1 DNA methylation in lymphocytes of methadone-maintained former heroin addicts, Neuropsychopharmacology 34 (4) (2009) 867–873, https://doi.org/10.1038/npp.2008.108.

[10] V.M. Chorbov, A.A. Todorov, M.T. Lynskey, T.J. Cicero, Elevated levels of DNA methylation at the OPRM1 promoter in blood and sperm from male opioid addicts, J. Opioid Manag. 7 (4) (2011) 258–264, https://doi.org/10.5055/jom.2011.0067.

[11] A. Groh, M. Rhein, V. Buchholz, A. Burkert, C.G. Huber, U.E. Lang, S.J. Borgwardt, A. Heberlein, M.A.N. Muschler, T. Hillemacher, S. Bleich, H. Frieling, M. Walter, Epigenetic effects of intravenous diacetylmorphine on the methylation of POMC and NR3C1, Neuropsychobiology 75 (4) (2017) 193–199, https://doi.org/10.1159/000486973.

[12] X.Y. Fan, G. Shi, P. Zhao, Reversal of oxycodone conditioned place preference by oxytocin: promoting global DNA methylation in the hippocampus, Neuropharmacology 160 (2019), 107778, https://doi.org/10.1016/j.neuropharm.2019.107778.

[13] F.Z. Jiang, W. Zheng, C. Wu, Y. Li, F. Shen, J. Liang, M. Li, J.J. Zhang, N. Sui, Double dissociation of inhibitory effects between the hippocampal TET1 and TET3 in the acquisition of morphine self-administration in rats, Addict. Biol. 7 (2020), e12875, https://doi.org/10.1111/adb.12875.

[14] P.J. Hamilton, E.J. Nestler, Epigenetics and addiction, Current Opinions in Neurobiology 59 (2019) 128–136, https://doi.org/10.1016/j.conb.2019.05.005.

[15] M. Ghazanfarpour, M.N. Najafi, N. Roozbeh, M.E. Mashhadi, A. Keramat-Roudi, B. Mégarbane, A. Tsatsakis, M.M.M. Moghaddam, R. Rezaee, Therapeutic approaches for neonatal abstinence syndrome: a systematic review of randomized clinical trials, Daru 27 (1) (2019) 423–431, https://doi.org/10.1007/s40199-019-00266-3.

[16] E.M. Wachman, L.A. Farrer, The genetics and epigenetics of neonatal abstinence syndrome, Semin. Fetal Neonatal Med. 24 (2) (2019) 105–110, https://doi.org/10.1016/j.siny.2019.01.002.

[17] G.Z. Papadakis, A.H. Karantanas, M. Tsiknakis, A. Tsatsakis, D.A. Spandidos, K. Marias, Deep learning opens new horizons in personalized medicine, Biomed. Rep. 10 (4) (2019) 215–217, https://doi.org/10.3892/br.2019.1199.

[18] J.K. Ahkin Chin Tai, J.L. Freeman, Zebrafish as an integrative vertebrate model to identify miRNA mechanisms regulating toxicity, Toxicol. Rep. 7 (2020) 559–570, https://doi.org/10.1016/j.toxrep.2020.03.010.

[19] A.M. Aloizou, G. Pateraki, V. Siokas, A.A. Mentis, I. Liampas, G. Lazopoulos, L. Kovatsi, P.D. Mitsias, D.P. Bogdanos, K. Paterakis, E. Dardiotis, The role of MiRNA-21 in gliomas: Hope for a novel therapeutic intervention? Toxicol. Rep. 7 (2020) 1514–1530, https://doi.org/10.1016/j.toxrep.2020.11.001.

[20] S. Balasubramanian, K. Gunasekaran, S. Sasidharan, V. Jeyamanickavel Mathan, E. Perumal, MicroRNAs and xenobiotic toxicity: an overview, Toxicol. Rep. 7 (2020) 583–595, https://doi.org/10.1016/j.toxrep.2020.04.010.

[21] B.N. Chorley, G.K. Carswell, G. Nelson, V.S. Bhat, C.E. Wood, Early microRNA indicators of PPARα pathway activation in the liver, Toxicol. Rep. 7 (2020) 805–815, https://doi.org/10.1016/j.toxrep.2020.06.006.

[22] S.C. Sahu, A. Tsatsakis, microRNAs: potential biomarkers of toxicity: a special issue of the journal Toxicology Reports, Toxicol. Rep. 7 (2020) 198–199, https://doi.org/10.1016/j.toxrep.2020.01.001.

[23] E. Schraml, M. Hackl, J. Grillari, MicroRNAs and toxicology: a love marriage, Toxicol. Rep. (2017) 634–636, https://doi.org/10.1016/j.toxrep.2017.11.001.

[24] R. Sisto, P. Capone, L. Cerini, F. Sanjust, E. Paci, D. Pigini, M.P. Gatto, M. Gherardi, A. Gordiani, N. L'Episcopo, G. Tranfo, P. Chiarella, Circulating microRNAs as potential biomarkers of occupational exposure to low dose organic solvents, Toxicol. Rep. 6 (2019) 126–135, https://doi.org/10.1016/j.toxrep.2019.01.001.

[25] R. Sisto, P. Capone, L. Cerini, E. Paci, D. Pigini, M. Gherardi, A. Gordiani, N. L'Episcopo, G. Tranfo, P. Chiarella, Occupational exposure to volatile organic compounds affects microRNA profiling: towards the identification of novel biomarkers, Toxicol. Rep. 7 (2020) 700–710, https://doi.org/10.1016/j.toxrep.2020.05.006.

[26] C.T. Werner, R.D. Altshuler, Y. Shaham, X. Li, Epigenetic mechanisms in drug relapse, Biol. Psychiatry S0006-3223 (20) (2020) 31836–31839, https://doi.org/10.1016/j.biopsych.2020.08.005. Advance online publication.

[27] American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders: DSM-IV, American Psychiatric Association, 1994.

[28] A.T. McLellan, L. Luborsky, J. Cacciola, J. Griffith, F. Evans, H.L. Barr, C. P. O'Brien, New data from the Addiction Severity Index. Reliability and validity in three centers, J. Nerv. Ment. Dis. 173 (7) (1985) 412–423, https://doi.org/10.1097/00005053-198507000-00005.

[29] World Medical Association Inc, Declaration of Helsinki. Ethical principles for medical research involving human subjects, J. Indian Med. Assoc. 107 (6) (2009) 403–405.

[30] C.W. Hu, J.L. Chen, Y.W. Hsu, C.C. Yen, M.R. Chao, Trace analysis of methylated and hydroxymethylated cytosines in DNA by isotope-dilution LC-MS/MS: first evidence of DNA methylation in Caenorhabditis elegans, Biochem. J. 465 (2015) 39–47, https://doi.org/10.1042/BJ20140844.

[31] C.W. Hu, H. Lee, J.L. Chen, Y.J. Li, M.R. Chao, Optimization of global DNA methylation measurement by LC-MS/MS and its application in lung cancer patients, Anal. Bioanal. Chem. 405 (2013) 8859–8869, https://doi.org/10.1007/s00216-013-7305-3.

[32] M.R. Chao, D. Fragou, P. Zanos, C.W. Hu, A. Bailey, S. Kouidou, L. Kovatsi, Epigenetically modified nucleotides in chronic heroin and cocaine treated mice, Toxicol. Lett. 229 (3) (2014) 451–457, https://doi.org/10.1016/j.toxlet.2014.07.023.