AUTHOR QUERY FORM

ELSEVIER	Journal: YMPD Article Number: 9340	Please e-mail your responses and any corrections to: E-mail: r.dalton@elsevier.com

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

We were unable to process your file(s) fully electronically and have proceeded by

☐ Scanning (parts of) your article ☐ Rekeying (parts of) your article ☐ Scanning the artwork

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	If there are any drug dosages in your article, please verify them and indicate that you have done so by initialing this query.
Q2	Please provide the grant number for 'Randox' if any.
Q3	Please confirm the accuracy of conflicts of interest and funding statements.
Q4	Please confirm that given names and surnames have been identified correctly.
Q5	Please check whether the affiliation(s) are correct.
Q6	Please include city and state (or country if not in the US) for Hewlett Packard .
Q7	Please check this website address and confirm that it is correct. (Please note that it is the responsibility of the author(s) to ensure that all URLs given in this article are correct and useable.)
Q8	The references should be sequentially cited in the text, hence refs have been renumbered both in the text and in the reference list from Ref. 18 (originally 30) onwards. Please check, and correct if necessary.
Q9	Table I: Expanded PCR to polymerase chain reaction. Please confirm.
	Please check this box or indicate your approval if you have no corrections to make to the PDF file ☐

Thank you for your assistance.

Increased DNA Methylation of *ABCB1*, *CYP2D6*, and *OPRM1* Genes in Newborn Infants of Methadone-Maintained Opioid-Dependent Mothers

Poppy McLaughlin, PhD[1], Helen Mactier, MBChB, MD, FRCPCH[2], Cheryl Gillis, MBChB[2], Tamas Hickish, MA, MD, FRCP[3], Anton Parker, BSc[3], Wei-Jun Liang, PhD[1], and M. David Osselton, PhD, FRCS[1]

Objective To investigate whether in utero opioid exposure, which has been linked to adverse neurodevelopmental and social outcomes, is associated with altered DNA methylation of opioid-related genes at birth.

Study design Observational cohort study of 21 healthy methadone-maintained opioid-dependent mother-infant dyads consecutively delivered at >36 weeks of gestation, and 2 comparator groups: smoking, "deprived" opioid-naïve mother-infant dyads (n = 17) and nonsmoking, "affluent" opioid-naïve mother-infant dyads (n = 15). DNA methylation of *ABCB1*, *CYP2D6*, and *OPRM1* genes for mothers and babies was determined from buccal swabs. Plasma methadone concentrations were additionally measured for methadone-maintained opioid-dependent mothers.

Results DNA methylation for *ABCB1* and *CYP2D6* was similar in opioid-naïve infants compared with their mothers, but was less for *OPRM1* (3 ± 1.6% vs 8 ± 1%, $P < .0005$). Opioid-exposed newborns had similar DNA methylation to their mothers for all genes studied and greater methylation of *ABCB1* (18 ± 4.8% vs 3 ± 0.5%), *CYP2D6* (92 ± 1.2% vs 89 ± 2.4%), and *OPRM1* (8 ± 0.3% vs 3 ± 1.6%) compared with opioid-naïve newborns ($P < .0005$ for all 3 genes). Infant DNA methylation was not related to birth weight, length of hospital stay, maternal smoking, dose or plasma concentration of methadone at delivery, or postcode of residence.

Conclusions In utero exposure to opioids is associated with increased methylation of opioid-related genes in the newborn infant. It is not clear whether these findings are due to opioid exposure per se or other associated lifestyle factors. *(J Pediatr 2017;■■:■■-■■).*

Methadone maintenance is the international standard of care for pregnant opioid dependent women.[1] Despite the advantages of methadone in stabilizing maternal lifestyle, there are problems associated with its use. Infants of methadone-maintained opioid-dependent (MMOD) mothers have shorter gestation periods, lower birth weights, smaller head circumferences, and are at risk of developing neonatal abstinence syndrome (NAS).[2,3] Visuocortical function is impaired at birth,[4] and the adverse effects of in utero opioid exposure continue into early childhood, adversely impacting upon visual development as well as cognitive, psychomotor, and behavioral performance.[5-7] It is not clear to what extent factors such as poverty and coexistent illicit drug and alcohol misuse contribute to adverse outcomes for infants of opioid-dependent mothers. Social and psychological problems may persist to adulthood, and there is some evidence of intergenerational substance misuse, particularly between substance misusing mothers and their daughters, although the mechanisms of this are poorly understood.[8,9]

Mechanisms by which opioids may influence fetal development include inhibition of neuronal proliferation and differentiation with increased cell death, alterations in endocrine function, and modifications to myelin sheath formations,[5,10] and it is feasible that fetal and later childhood outcomes of opioid-exposed pregnancies are influenced by in utero changes to DNA methylation.[11]

Increased DNA methylation on the μ-opioid receptor gene (*OPRM1*) in sperm and white blood cells in adult subjects has been attributed to opioid misuse,[12-14] and has also been described in relation to the development of NAS in methadone-exposed newborns.[15] To date no studies have reported DNA methylation of opioid-related genes in infants of MMOD mothers compared with opioid-naïve infants.

The aims of this study were to compare differences in DNA methylation on selected opioid-related genes (*ABCB1*, *CYP2D6*, and *OPRM1*) between MMOD mothers and their newborn infants and opioid-naive mothers and their newborn infants, and to examine whether DNA methylation in the newborn is associated with in utero growth, development of NAS, or length of hospital stay. We also sought

CpG	Cytosine-phosphate-guanine dinucleotide
DEPCAT	Deprivation score
MMOD	Methadone-maintained opioid-dependent
NAS	Neonatal abstinence syndrome

From the [1]Department of Archaeology, Anthropology, and Forensic Science, Bournemouth University, Poole; [2]Neonatal Unit, Princess Royal Maternity, Glasgow; and [3]Department of Molecular Pathology, Royal Bournemouth Hospital, Bournemouth, United Kingdom

Funded by Randox (Co. Antrim, Northern Ireland). The Royal Bournemouth Hospital (Dorset, United Kingdom) provided the work space for genetic analysis. The authors declare no conflicts of interest.

http://dx.doi.org10.1016/j.jpeds.2017.07.026

to investigate whether maternal cigarette smoking or postcode of resident (as a proxy for socioeconomic deprivation) influence DNA methylation in the newborn.

Methods

All MMOD mothers delivering after 36 completed weeks of gestation at Princess Royal Maternity in Glasgow were eligible to participate in the study. Potential subjects were identified in the postnatal wards, soon after delivery. These mothers had been managed within an established multidisciplinary service for women with social problems including substance misuse; antenatal care included ongoing methadone maintenance provided in collaboration with social work and addiction services and tailored to symptoms. Sufficient methadone was prescribed to eliminate physical withdrawals, with the aim of reducing toward the lowest acceptable dose of methadone in the weeks before delivery. Exclusion criteria included unwell babies and those born before 36 completed weeks of gestation.

Within 24-72 hours of delivery and following informed parental consent, a buccal swab (Catch-All; Cambio Ltd, Cambridge, United Kingdom) was obtained from the baby and a venous blood sample obtained from the mother for estimation of trough plasma methadone concentration. The latter was drawn shortly before administration of the once daily prescribed dose of methadone. Demographic data including maternal age, prescribed methadone dose at delivery, smoking status, and postcode of residence were extracted from case records. Deprivation score (DEPCAT) was calculated from postcode using Carstairs index.[16] Use of other drugs (illicit or prescribed) was determined from case records and from individual discussion with mothers as well as from urine samples immediately postnatally, when available. Routine antenatal urine toxicology was not hospital policy and, thus, was not included in this study.

Infant gestation, birth weight, and length of hospital stay were also recorded from case records. All babies were nursed in the postnatal ward with their mothers and NAS was managed according to protocol; scoring used a local version of the Lipsitz scale.[17] Infants scoring 5 or more on 2 consecutive occasions and/or with poor feeding or ongoing weight loss after 5 days were commenced on oral morphine at a dose of 60 μg/kg 6 times daily. Treatment was escalated to 80 μg/kg/dose if the baby remained symptomatic otherwise morphine was weaned daily by 10 μg/kg/dose. If NAS symptoms were not controlled by oral morphine, phenobarbital was given in addition. Regardless of treatment, all infants remained with their mother in hospital for a minimum of 5 days. Length of stay for treated babies was determined by success of weaning of morphine; for treatment periods greater than 10-12 days, the mother was discharged from hospital and baby admitted to the neonatal unit. Following weaning of oral morphine, phenobarbital treatment could be continued as an outpatient. Breast feeding was encouraged for all babies. The research team was not involved in any decision to treat an infant.

To control for the effects of cigarette smoking and poverty on DNA methylation, 2 groups of nonopioid dependent mother-infant dyads were recruited from the postnatal wards of the same maternity hospital, based on maternal smoking (yes or no) and postcode of residence. Cigarette smoking mothers from DEPCAT scores 4-7 comprised a "deprived" group and nonsmoking mother-infant dyads from more affluent areas of Glasgow (DEPCAT 1-3) an "affluent" group. Oral swabs were obtained from opioid-naïve mothers and infants within 24-48 hours of delivery and maternal age, infant birth weight, and gestation recorded. Infant buccal swabs were collected before commencement of treatment for NAS. All mothers and babies were of Caucasian origin.

Limited funding allowed for investigation of 50 mother-infant dyads in this pilot study; we aimed, therefore, to recruit 20 MMOD and 15 each deprived and affluent nonopioid-exposed dyads. The study was approved by West of Scotland Research Ethics Committee 5, and all mothers gave informed, written consent.

Methylation Analysis

DNA extracted from buccal swabs underwent bisulfite conversion using the EZ DNA Methylation-Gold kit protocol (Zymo Research, Frieburg, Germany) and was amplified by a Veriti Thermal Cycler (Life Technologies, Carlsbad, California). Percentage methylation was quantified using the Q24 Pyrosequencer (Qiagen, Hilden, Germany). Details of the primers used are listed in **Table I** (available at www.jpeds.com). The ABCB1 gene primers amplify the −356 bp to −265 bp region relative to the ATG start codon that contains 11 cytosine-phosphate-guanine dinucleotide (CpG) sites. Three CpG sites of CYP2D6 gene investigated. The 2 regions of *OPRM1* amplified (promoter (−30 bp to −7 bp) and exon 1 (+12 bp to +27 bp)) contain 8 CpG sites of interest.

Methadone Analysis

Methadone was isolated from 1 mL of plasma at the Toxicology Unit, Imperial College, London by liquid-liquid extraction, using clomipramine-$_{D3}$ as the internal standard. Quantification was undertaken using a Hewlett Packard 6890 gas chromatograph linked to a 5973 mass spectrometer.

Statistical Analyses

Statistical analyses were conducted using SPSS v 23 (SPSS Inc, Chicago, Illinois). Maternal age, gestation, birth weight, and gene methylation differences between the opioid-exposed and naïve mother, and infant populations were determined using 1-way ANOVA, as were the differences between gene methylation and requirement for NAS treatment. The data were interrogated for outliers by visually inspecting box plots, and normality was assessed using the Shapiro-Wilk test. If either of these tests were violated, the data were transformed. If transformation of the data did not produce normal distribution, Welch and Games-Howell post-hoc tests were undertaken. For normally distributed data with no outliers, Tukey post-hoc tests were carried out.

FLA 5.5.0 DTD ■ YMPD9340_proof ■ July 28, 2017

Table II. Mother and infant demographics

	MMOD (n = 21)	Deprived (n = 17)	Affluent (n = 15)	*P*
Maternal age (y, mean ± SD)	32 ± 4	28 ± 6	33 ± 4	.01
Maternal smoking (n, %)	21 (100)	16 (94)	0 (0)	—
Gestation (wk, mean ± SD)	39 ± 1	40 ± 1	39 ± 2	.15
Birth weight (g, mean ± SD)	2815 ± 353	3364 ± 549	3413 ± 533	<.01

Results

Twenty-one of 22 consecutively delivered MMOD mothers approached agreed to participate in the study, and samples were obtained from all 21 mother-infant dyads. In addition, 17 deprived and 15 affluent (opioid-naïve) mother-infant dyads were recruited. Affluent and MMOD mothers were of similar age (median 32 years); deprived mothers were younger (28 years, $P = .02$) (Table II). Gestation ranged from 36^{+1} to 42^{+0} (mean 38^{+6}) weeks and did not differ between opioid-exposed and opioid-naïve infants born in either affluent or deprived postcodes. Birth weight did not differ between affluent and deprived controls but was lower in the MMOD group than combined controls (2815 ± 352.5 g vs 3386 ± 533.7 g, $P < .0005$). Median hospital stay for infants of MMOD mothers was 11 days (range 5-42 days); opioid-naïve infants were discharged home with their mothers within 24-72 hours after birth, depending on mode of delivery.

MMOD mothers had been prescribed a mean daily methadone dose of 59 ± 27.6 mg at delivery. Plasma samples were obtained from 18 of 21 MMOD mothers; median methadone plasma concentration was 245 (range 60-720 μg/L). In addition to prescribed maintenance methadone, all MMOD mothers had smoked cigarettes, and a majority admitted to consumption of other drugs, including heroin (smoked or injected), prescription and/or illicit benzodiazepines, and cannabis. Eleven of 21 infants born to MMOD mothers required treatment for NAS; 2 of these treated babies additionally required phenobarbital.

There was little variation in methylation between CpG sites for each of the genes studies, and so averaged results were used.

ABCB1

ABCB1 methylation was assessed for 20 opioid-naïve infants, 9 opioid-naïve mothers, 19 opioid-exposed infants, and 18 MMOD mothers. *ABCB1* methylation was the same in opioid-naïve mothers and infants, and in opioid-exposed mothers and their infants, but was significantly higher in the opioid-exposed mother-infant dyads, compared with opioid-naïve subjects (3 ± 0.5% in opioid naïve newborns; 3 ± 0.4% in opioid naïve mothers; 18 ± 4.8% in opioid exposed newborns; 16 ± 8.3% in MMOD mothers (Welch $F[3, 28.174] = 56.031$, $P < .0005$) (Figure).

CYP2D6

CYP2D6 methylation was assessed for 32 opioid-naïve infants and their mothers and for 18 MMOD mothers and their infants. There were no differences between opioid-naïve or opioid-exposed infants and their mothers (Figure). *CYP2D6* methylation was marginally but significantly higher in MMOD mothers (92 ± 1.7%) and their infants (92 ± 1.2%) compared with opioid-naïve mothers (89 ± 4.2%) and their infants (89 ± 2.4%) (Welch F [3, 48083] = 14.755, $P < .0005$).

OPRM1

OPRM1 methylation was assessed for 30 opioid-naïve infants, 29 opioid-naïve mothers, 20 opioid-exposed infants, and 19 MMOD mothers. *OPRM1* methylation was significantly lower in opioid-naïve infants (3 ± 1.6%) compared with in opioid-naïve mothers (8 ± 2.0%), opioid-exposed infants (8 ± 0.3%),

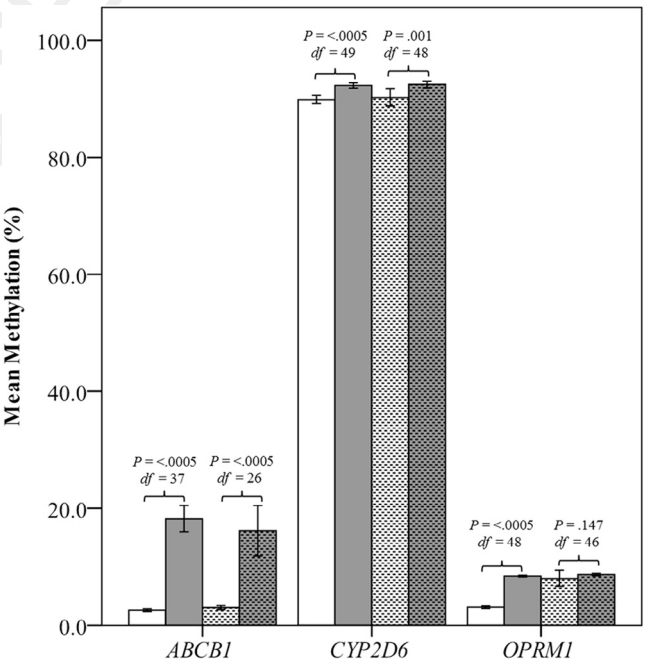

Figure. *ABCB1*, *CYP2D6*, and *OPRM1* gene DNA methylation in MMOD mothers and their newborns compared with opioid-naïve mothers and newborns. Opioid-exposed newborns had higher % methylation than opioid naive newborns on all genes investigated. Similar methylation was observed on the *OPRM1* gene between MMOD and opioid naïve mothers, however, methylation was higher on *ABCB1* and *CYP2D6* in MMOD mothers. *White filled column* = opioid-naïve newborns; *solid grey column* = opioid-exposed newborns; *checkered white filled column* = opioid-naive mothers; *checkered gray filled column* = MMOD mothers.

FLA 5.5.0 DTD ■ YMPD9340_proof ■ July 28, 2017

Table III. Relationship between gene methylation and development of NAS requiring treatment (opioid-exposed newborns only)

		ABCB1 (%)	*P*	*CYP2D6* (%)	*P*	*OPRM1* (%)	*P*
Treated NAS	Range	12.9-23.1	0.3	91.0-93.9	0.9	8.0-9.0	1.0
n = 10	Mean ± SD	18.9 ± 2.8		92.0 ± 1.1		8.5 ± 0.3	
No NAS	Range	4.8-23.3		88.9-93.5		8.2-8.8	
n = 11	Mean ± SD	16.6 ± 6.4		92.1 ± 9.4		8.5 ± 0.2	

and in MMOD mothers (9 ± 0.1%). (Games-Howell post hoc analysis; −4.61 (95% CI −5.90 to −3.33; $P < .0005$), 4.99 (95% CI −5.81 to −4.17; $P < .0005$), and −5.20 (95% CI −6.03 to −4.36; $P < .0005$), respectively. (**Figure**).

There was no relationship between infant DNA methylation of any of the genes studied and either birth weight, length of hospital stay, maternal dose, or plasma concentration of methadone. DNA methylation was not different between opioid-exposed newborns who did or did not require treatment for NAS (**Table III**).

Discussion

We explored DNA methylation on *ABCB1* and *CYP2D6* genes in methadone exposed newborns, and also compared DNA methylation between opioid-exposed and opioid-naïve newborns. We found DNA methylation of all 3 opioid-related genes in newborn infants of MMOD mothers to be similar to that of their mothers, and higher than that of opioid-naïve newborns. Increased methylation on *OPRM1* is consistent with the study of Wachman et al[15] who analyzed a larger population (n = 86) of infants exposed to maternal opioids, 65% of whom required pharmacologic treatment for NAS. Our failure to confirm an association between increased methylation of the *OPRM1* promoter and severity of NAS may well be a type II error, reflective of our smaller study numbers.

Opioid misuse is associated with numerous lifestyle factors deleterious to health. We did not have accurate toxicology data to confirm additional substance use, but from previous studies in a very similar population, we assume that this was common and most likely to be illicit opioids and benzodiazepines.[2] Prenatal exposures to antidepressants, antiepileptic drugs, alcohol, antibiotics, and tobacco have been associated with altered neonatal DNA methylation.[24] We did not see an effect of cigarette smoking upon DNA methylation in opioid-naïve newborns, but study numbers were small. Socioeconomic deprivation may also have contributed to increased DNA methylation in opioid-exposed mother-infant dyads.[25] We did not find any differences between opioid-naïve "deprived" and "affluent" dyads, but we acknowledge that numbers were small, and that postcode of residence is a poor proxy for social deprivation. Besides small patient numbers, a limitation of the present study is that the nutritional intake of mothers was not obtained. It is, therefore, not possible to conclude if the increased DNA methylation of *ABCB1*, *CYP2D6*, and *OPRM1* genes observed in opioid-exposed newborns was due to maternal prescribed substitute methadone, ongoing illicit drug use, or other lifestyle factors associated with drug misuse.

Disparity in gene methylation between opioid-exposed and opioid-naïve infants could have originated during any stage of fetal development. Fertilization and early embryogenesis is a particularly sensitive period of epigenetic reprograming when paternal and maternal genomes are actively and passively demethylated to facilitate the conversion from somatic to germline epigenotype that enables cell differentiation.[26] A plausible mechanism by which methadone could increase DNA methylation is through G-protein-coupled receptor-mediated increase of DNA methyltransferase activity,[14] mediated by activation of mitogen-activated protein kinase-dependent pathways.[27,28]

Higher maternal DNA methylation on *ABCB1* and *OPRM1* observed in opioid-naïve dyads would be predicted as a result of age associated epigenetic alterations of CpG rich regions,[29] whereas the lack of difference between opioid-naïve mothers and infant *CYP2D6* gene methylation could be a tissue specific phenomenon. *CYP2D6* gene expression is not necessary in buccal DNA and may not reflect methylation of brain DNA.

The association of hypermethylation with methadone treatment suggests interaction between opioids and mechanisms of classical epigenetics.[30] Global DNA methylation analysis[18] would have addressed whether our observations reflect a global increase in DNA methylation rather than a specific effect upon a few genes but was beyond the scope and purpose of this particular study.

DNA methylation of *ABCB1*, *CYP2D6*, and *OPRM1* opioid-related genes is increased in newborn infants of MMOD mothers, but the mechanisms of this are not clear. The effect of this phenomenon should be explored in relation to the infant's behavior and susceptibility to disease and drug use later in life.

Availability of Data and Materials

The datasets and analyses are available from the corresponding author on reasonable request. ■

The authors thank Susan Paterson and Rosa Codero for help with sample analyses and Dr Andrew Whittington for statistical advice. None of these persons received any funding in relation to this study, and all declare no conflict of interest.

Submitted for publication Feb 20, 2017; last revision received Jun 5, 2017; accepted Jul 13, 2017

Reprint requests: Helen Mactier, MBChB, MD, FRCPCH, Neonatal Unit, Princess Royal Maternity, 8–16, Alexandra Parade, Glasgow G31 2ER, United Kingdom. E-mail: helen.mactier@ggc.scot.nhs.uk

References

1. Mattick RP, Breen C, Kimber J, Davoli M. Buprenorphine maintenance versus placebo or methadone maintenance for opioid dependence. Cochrane Database Syst Rev 2014;2:CD002207. doi:10.1002/14651858.CD002207.pub4.
2. Dryden C, Young D, Hepburn M, Mactier H. Maternal methadone use in pregnancy: factors associated with the development of neonatal abstinence syndrome and implications for healthcare resources. BJOG 2009;116:665-71.
3. Cleary BJ, Donnelly JM, Strawbridge JD, Gallagher PJ, Fahey T, White MJ, et al. Methadone and perinatal outcomes: a retrospective cohort study. Am J Obstet Gynecol 2011;139:e1-9.
4. McGlone L, Hamilton R, McCulloch DL, Boulton R, Bradnam MS, Weaver LT, et al. Neonatal visual evoked potentials are altered in infants born to mothers prescribed methadone in pregnancy. Pediatrics 2013;131:e857-63.
5. Konijnenberg C, Melinder A. Neurodevelopmental investigation of the mirror neurone system in children of women receiving opioid maintenance therapy during pregnancy. Addiction 2013;108:154-60.
6. Baldacchino AM, Arbuckle K, Petrie DJ, McCowan C. Erratum: neurobehavioral consequences of chronic intrauterine opioid exposure in infants and preschool children: a systematic review and meta-analysis. BMC Psychiatry 2015;15:134.
7. McGlone L, Hamilton R, Mackinnon J, Bradnam MS, McCulloch DL, Mactier H. Visual outcome in infants born to drug-misusing mothers prescribed methadone in pregnancy. Br J Ophthalmol 2014;98:238-45.
8. Herranz GS, Vílchez MAL, Ledo JD, Sierra AM. Children born to heroin-addicted mothers: what's the outcome 25 years later? J Addict Res Ther 2014;5:180.
9. Thornberry TP, Krohn M, Freeman-Gallant A. Intergenerational roots of early onset substance use. J Drug Issues 2006;38:1-28.
10. Farid WO, Dunlop SA, Tait RJ, Hulse GK. The effects of maternally administered methadone, buprenorphine and naltrexone on offspring: review of human and animal data. Curr Neuropharmacol 2008;6:125-50.
11. Geiman TM, Muegge K. DNA methylation in early development. Mol Reprod Dev 2010;77:105-13.
12. Nielsen DA, Yuferov V, Hamon S, Jackson C, Ho A, Ott J, et al. Increased OPRM1 DNA methylation in lymphocytes of methadone-maintained former heroin addicts. Neuropsychopharmacology 2009;34:867-73.
13. Chorbov VM, Todorov AA, Lynskey MT, Cicero TJ. Elevated levels of DNA methylation at the OPRM1 promoter in blood and sperm from male opioid addicts. J Opioid Manag 2010;7:258-64.
14. Doehring A, Oertel BG, Sittl R, Lötsch J. Chronic opioid use is associated with increased DNA methylation correlating with increased clinical pain. Pain 2013;154:15-23.
15. Wachman EM, Hayes MJ, Lester BM, Terrin N, Brown MS, Nielsen DA, et al. Epigenetic variation in the mu-opioid receptor gene in infants with neonatal abstinence syndrome. J Pediatr 2014;165:472-8.
16. McLoone P. Carstairs scores for Scottish postcode sectors from the 2001 Census. MRC Glasgow: Social and Public Health Sciences Unit, University of Glasgow, 2004. http://www.msoc-mrc.gla.ac.uk. Accessed June 20, 2016.
17. Lipsitz PJ. A proposed narcotic withdrawal score for use with newborn infants. A pragmatic evaluation of its efficacy. Clin Pediatr (Phila) 1975;14:592-4.
18. Dejeux E, Rønneberg JA, Solvang H, Bukholm I, Geisler S, Aas T, et al. Research DNA methylation profiling in doxorubicin treated primary locally advanced breast tumours identifies novel genes associated with survival and treatment response. Mol Cancer 2010;9:68-81.
19. Gurnot C, Martin-Subero I, Mah SM, Weikum W, Goodman SJ, Brain U, et al. Prenatal antidepressant exposure associated with CYP2E1 DNA methylation change in neonates. Epigenetics 2015;10:361-72.
20. Smith AK, Conneely KN, Newport DJ, Kilaru V, Schroeder JW, Pennell PB, et al. Prenatal antiepileptic exposure associates with neonatal DNA methylation differences. Epigenetics 2012;7:458-63.
21. Masemola ML, van der Merwe L, Lombard Z, Viljoen D, Ramsay M. Reduced DNA methylation at the PEG3 DMR and KvDMR1 loci in children exposed to alcohol in utero: a South African Fetal Alcohol Syndrome cohort study. Front Genet 2015;6:85.
22. Vidal AC, Murphy SK, Murtha AP, Schildkraut JM, Soubry A, Huang Z, et al. Associations between antibiotic exposure during pregnancy, birth weight and aberrant methylation at imprinted genes among offspring. Int J Obes (Lond) 2013;37:907-13.
23. Suter M, Ma J, Harris AS, Patterson L, Brown KA, Shope C, et al. Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression. Epigenetics 2011;6:1284-94.
24. Joubert BR, Håberg SE, Bell DA, Nilsen RM, Vollset SE, Midttun O, et al. Maternal smoking and DNA methylation in newborns: in utero effect or epigenetic inheritance? Cancer Epidemiol Biomarkers Prev 2014;23:1007-17.
25. De Rooij SR, Costello PM, Veenendaal MV, Lillycrop KA, Gluckman PD, Hanson MA, et al. Associations between DNA methylation of a glucocorticoid receptor promoter and acute stress responses in a large healthy adult population are largely explained by lifestyle and educational differences. Psychoneuroendocrinology 2012;37:782-8.
26. Rivera RM, Ross JW. Epigenetics in fertilization and preimplantation embryo development. Prog Biophys Mol Biol 2013;113:423-32.
27. Paradisi A, Pasquariello N, Barcaroli D, Maccarrone M. Anandamide regulates keratinocyte differentiation by inducing DNA methylation in a CB1 receptor-dependent manner. J Biol Chem 2008;83:6005-12.
28. Morse M, Tran E, Sun H, Levenson R, Fang Y. Ligand-directed functional selectivity at the mu opioid receptor revealed by label-free integrative pharmacology on-target. PLoS ONE 2011;6:e25643.
29. Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. Nat Rev Genet 2012;13:679-92.
30. Knothe C, Doehring A, Ultsch A, Lötsch J. Methadone induces hypermethylation of human DNA. Epigenetics 2016;8:167-79.

Table I. Primers for polymerase chain reaction and pyrosequencing

Genes (region amplified)	PCR primers* (no. of cycles, annealing temperature)	Pyrosequencing primers
ABCB1 (promoter)	F:AAAACAAAATTAAAAATCTAACAAC R:Bio-TTAGATTTAGGAGTTTTTGGAGTAG (50, 57°C)	Seq 1: TGGTATTGGATTATGTTGTT Seq 2: TGGGTGGGAGGAAGT
CYP2D6 (exon 1)	F:Bio-TGGAGTAGGAAGTAGGGGTAAGAAT R:AACACAAAAAACCAAACAAAACAC (35, 58.5°C)	AAACACTCTCAACACACC
OPRM1 (promoter)	F:GGATTGGTTTTTGTAAGAAATAGTA R:Bio-CTAAAAACAACCCTACTATCCATAATA (35, 50°C)	AGTTTAGGTGTTTTTGGTTA
OPRM1 (exon 1)	F:Bio-GGATTGGTTTTTGTAAGAAATAGTA R:CTAAAAACAACCCTACTATCCATAATAC (35, 58°C)	CCAAAACATCAATACAATTA

Bio, biotin labeled; *PCR*, polymerase chain reaction.
**ABCB1* primers designed by Dejeux et al[18] were used; primers for CYP2D6 and OPRM1 were designed in-house.

FLA 5.5.0 DTD ■ YMPD9340_proof ■ July 28, 2017