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.

<table>
<thead>
<tr>
<th>Location in article</th>
<th>Query / Remark: click on the Q link to go</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>If there are any drug dosages in your article, please verify them and indicate that you have done so by initialing this query.</td>
</tr>
<tr>
<td>Q2</td>
<td>Please provide the grant number for 'Randox' if any.</td>
</tr>
<tr>
<td>Q3</td>
<td>Please confirm the accuracy of conflicts of interest and funding statements.</td>
</tr>
<tr>
<td>Q4</td>
<td>Please confirm that given names and surnames have been identified correctly.</td>
</tr>
<tr>
<td>Q5</td>
<td>Please check whether the affiliation(s) are correct.</td>
</tr>
<tr>
<td>Q6</td>
<td>Please include city and state (or country if not in the US) for Hewlett Packard.</td>
</tr>
<tr>
<td>Q7</td>
<td>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.)</td>
</tr>
<tr>
<td>Q8</td>
<td>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.</td>
</tr>
<tr>
<td>Q9</td>
<td>Table I: Expanded PCR to polymerase chain reaction. Please confirm.</td>
</tr>
</tbody>
</table>

Please check this box or indicate your approval if you have no corrections to make to the PDF file

Thank you for your assistance.

CE: RB
Increased DNA Methylation of \( \text{ABCB1}, \ \text{CYP2D6}, \ \text{and OPRM1} \) Genes in Newborn Infants of Methadone-Maintained Opioid-Dependent Mothers

**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-naive mother-infant dyads (n = 17) and nonsmoking, “affluent” opioid-naive mother-infant dyads (n = 15). DNA methylation of \( \text{ABCB1}, \ \text{CYP2D6}, \ \text{and OPRM1} \) 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 \( \text{ABCB1} \) and \( \text{CYP2D6} \) was similar in opioid-naive infants compared with their mothers, but was less for \( \text{OPRM1} \) (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 \( \text{ABCB1} \) (18 ± 4.8% vs 3 ± 0.5%), \( \text{CYP2D6} \) (92 ± 1.2% vs 89 ± 2.4%), and \( \text{OPRM1} \) (8 ± 0.3% vs 3 ± 1.6%) compared with opioid-naive 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;■■■■─■■).*

**Methodology**

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,\(^1\) and the adverse effects of 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 changes to DNA methylation.\(^11\)

Increased DNA methylation on the \( \mu \)-opioid receptor gene (\( \text{OPRM1} \)) 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-naive infants.

The aims of this study were to compare differences in DNA methylation on selected opioid-related genes (\( \text{ABCB1}, \ \text{CYP2D6}, \ \text{and OPRM1} \)) 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...
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. 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. 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.

**Methylation Analysis**

DNA extracted from buccal swabs underwent bisulfite conversion using the EZ DNA Methylation-Gold kit protocol (Zymo Research, 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 primers amplified 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 were 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-3H 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.
Table II. Mother and infant demographics

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

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-naive) 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 to 42 (mean 38± weeks and did not differ between opioid-exposed and opioid-naive 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-naive 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 studied, and so averaged results were used.

ABCB1

ABCB1 methylation was assessed for 20 opioid-naive infants, 9 opioid-naive mothers, 19 opioid-exposed infants, and 18 MMOD mothers. ABCB1 methylation was the same in opioid-naive 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-naive subjects (3 ± 0.5% in opioid naive newborns; 3 ± 0.4% in opioid naive 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-naive infants and their mothers and for 18 MMOD mothers and their infants.

There were no differences between opioid-naive 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-naive 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-naive infants, 29 opioid-naive mothers, 20 opioid-exposed infants, and 19 MMOD mothers. OPRM1 methylation was significantly lower in opioid-naive infants (3 ± 1.6%) compared with opioid-naive mothers (8 ± 2.0%), opioid-exposed infants (8 ± 0.3%), opioid-naive mothers (92 ± 1.7%), and their infants (92 ± 1.2%).
and in MMOD mothers (9 ± 0.1%). % Change–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 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. Prenatal exposures to antidepressants, antiepileptic drugs, alcohol, antibiotics, and tobacco had all been associated with altered neonatal DNA methylation. 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. 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 reprogramming when paternal and maternal genomes are actively and passively demethylated to facilitate the conversion from somatic to germline epigenotype that enables cell differentiation. A plausible mechanism by which methadone could increase DNA methylation is through G-protein-coupled receptor-mediated increase of DNA methyltransferase activity, mediated by activation of mitogen-activated protein kinase-dependent pathways.

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, whereas the lack of difference between opioid-naïve mother 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. Global DNA methylation analysis 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
References


13. Chorbov VM, Todorov AA, Lyskey MT, Ciceri 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.


<table>
<thead>
<tr>
<th>Genes (region amplified)</th>
<th>PCR primers* (no. of cycles, annealing temperature)</th>
<th>Pyrosequencing primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1 (promoter)</td>
<td>F:AAAACAAAATTTAATCAACAC R:Bio-TTAGATTAGGAGTTTGTGAGTAG (50, 57°C)</td>
<td>Seq 1: TGGTATTAGATGTGGTGTGT Seq 2: TGGGTTGGAGGAAAGT</td>
</tr>
<tr>
<td>CYP2D6 (exon 1)</td>
<td>F:Bio-TGAGATGGAGGTGGTGAAGAAT R:AACACAAAAACCAAAGACAC (35, 58.5°C)</td>
<td>AAACACTCTCAACACACC</td>
</tr>
<tr>
<td>OPRM1 (promoter)</td>
<td>F:GGATTGGTTTTTGTAAAGAATAGTA R:Bio-CTAATGTCTGTAATCTACAAATATAC (35, 50°C)</td>
<td>AGTTTGAATGTGTTTTGTT</td>
</tr>
<tr>
<td>OPRM1 (exon 1)</td>
<td>F:Bio-GGATTGGTTTTTGTAAAGAATAGTA R:CTAATAACACCCCTAATCTACAAATAC (35, 58°C)</td>
<td>CCAAAACATCAATCAATTA</td>
</tr>
</tbody>
</table>

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