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

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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 the 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.

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* 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 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 (Hewlett Packard, Avondale, Pennsylvania).

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.

Results

Twenty-one of 22 consecutively delivered MMOD mothers approached agreed to participate in the study, and samples were

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