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Epigenetic Variation in the Mu-opioid Receptor Gene in Infants with Neonatal Abstinence Syndrome

Elisha M Wachman, MD¹, Marie J Hayes, PhD², Barry M Lester, PhD³, Norma Terrin, PhD^{4,8}, Mark S Brown, MD⁵, David A Nielsen, PhD⁶, and Jonathan M Davis, MD^{4,7}

¹Pediatrics, Boston Medical Center, Boston, MA

²Psychology, Graduate School of Biomedical Sciences and Engineering, University of Maine, Orno, ME

³Center for the Study of Children at Risk, Alpert Medical School of Brown University and Women and Infant's Hospital, Providence, RI

⁴Tufts Clinical and Translational Science Institute, Tufts University, Boston, MA

⁵Pediatrics, Eastern Maine Medical Center, Bangor, ME

⁶Menninger Department of Psychiatry and Behavioral Sciences, Baylor College of Medicine, Houston, TX

⁷Pediatrics, The Floating Hospital for Children at Tufts Medical Center, Boston, MA

⁸Institute for Clinical Research and Health Policy Studies, Tufts Medical Center, Boston, MA

Abstract

Objective—Neonatal abstinence syndrome (NAS) from *in utero* opioid exposure is highly variable with genetic factors appearing to play an important role. Epigenetic changes in cytosine:guanine (CpG) dinucleotide methylation can occur after drug exposure and may help to explain NAS variability. We correlated DNA methylation levels in the mu-opioid receptor (*OPRM1*) promoter in opioid-exposed infants and correlate them with NAS outcomes.

Study design—DNA samples from cord blood or saliva were analyzed for 86 infants being treated for NAS according to institutional protocol. Methylation levels at 16 *OPRM1* CpG sites were determined and correlated with NAS outcome measures, including need for treatment, treatment with >2 medications, and length of hospital stay. We adjusted for co-variables and multiple genetic testing.

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Corresponding Author: Elisha Wachman, MD, Department of Pediatrics, Boston Medical Center, 771 Albany Street, Dowling 4N 4109, Boston, MA 02118, Elisha.Wachman@bmc.org, Phone: 617-548-8858, Fax: 617-414-7297.

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Results—Sixty-five percent of infants required treatment for NAS, and 24% required 2 medications. Hypermethylation of the *OPRM1* promoter was measured at the -10 CpG in treated versus non-treated infants [adjusted difference $\delta=3.2\%$ (95% CI 0.3–6.0%), $p=0.03$; NS after multiple testing correction]. There was hypermethylation at the -14 [$\delta=4.9\%$ (95% CI 1.8–8.1%), $p=0.003$], -10 [$\delta=5.0\%$ (95% CI 2.3–7.7%), $p=0.0005$], and +84 [$\delta=3.5\%$ (95% CI 0.6 – 6.4), $p=0.02$] CpG sites in infants requiring 2 medications which remained significant for -14 and -10 after multiple testing correction.

Conclusions—Increased methylation within the *OPRM1* promoter is associated with worse NAS outcomes, consistent with gene silencing.

Keywords

DNA methylation; NAS; opioids; genetics; *OPRM1*

Neonatal abstinence syndrome (NAS), a constellation of signs and symptoms due to withdrawal from *in-utero* opioid exposure is a growing problem, now affecting 5.6 per 1000 births.(1–2) The incidence of NAS has tripled in the past decade, affecting 60–80% of infants born to mothers on methadone, buprenorphine, or other prescription narcotics.(1) NAS is associated with long hospitalizations, extensive pharmacological therapy and variable newborn recovery with increased healthcare costs.(3–4) Much of what influences the variability in the incidence and severity of NAS remains unknown, with genetic factors appearing to be important.(5–7)

Genetic factors contribute to an individual's risk for opiate addiction, with candidate genes identified as modulators of opioid therapy in dependent adults.(8–9) Specifically, the mu-opioid receptor gene *OPRM1* is the primary site of action of endogenous and exogenous opioids. A number of studies have associated single-nucleotide polymorphisms (SNPs) in this gene with an increased risk for substance abuse in adults.(9–12) Common variants such as the 118A>G rs1799971 SNP are known to have functional consequences.(11–12) In the first study examining genetic variants in infants with NAS, infants with the *OPRM1* rs1799971 AG or GG genotype had improved NAS outcomes compared with infants with the AA genotype.(6)

In addition to changes in the DNA sequence, changes in gene expression due to epigenetic modifications may influence NAS. Epigenetic changes are important in adults and triggered by the use of an addictive drug, leading to drug cravings and a diminished response to pharmacotherapy.(13) Cytosine methylation of DNA is a common epigenetic mechanism that occurs through the addition of a methyl group to the cytosine residues of cytosine:guanine (CpG) dinucleotides. Chronic opioid exposure may lead to modifications of methylation levels at specific CpG sites within promoter regions of a gene, potentially leading to an increase or decrease in gene expression.(13–14) Prior studies of *OPRM1* have demonstrated that an increase in promoter methylation is associated with a decrease in protein expression of the mu-opioid receptor.(15) In addition, hypermethylation at selected CpG sites within *OPRM1* was present in opioid dependent adults but not in control individuals.(16–18) These changes have also been identified in sperm of opioid dependent males, suggesting heritability.(16)

Epigenetic changes in *OPRM1* have not been examined in NAS. Variability in the severity of NAS may be dependent on different methylation patterns, thus influencing opioid receptor system responsiveness to opioids. The purpose of this study is to examine CpG methylation patterns within the *OPRM1* promoter region in infants chronically exposed to *in-utero* opioids and to correlate these epigenetic changes with NAS outcome measures.

METHODS

Eighty-six infants 36 weeks gestational age were enrolled at Tufts Medical Center and affiliated nurseries (Brockton Hospital, Melrose Wakefield Hospital, and Lowell General Hospital) and Eastern Maine Medical Center (EMMC) between 2011 and 2012. This study had the same infant DNA samples and dataset from a previously published study examining SNP genotype in the *OPRM1* gene in infants with NAS.(6) Eligibility criteria included maternal prescribed methadone or buprenorphine exposure *in-utero* for at least 30 days prior to delivery, singleton pregnancies, and infants who were medically stable after delivery without other significant complications. The study was approved by the Institutional Review Boards of all sites with written informed consent.

DNA was sampled from either cord blood (PAXgene Blood DNA tube) or saliva (Oragene OG-250 DNA collection kit with CS-1 sponges).(19–20) If a cord blood sample was not available at the time of delivery, a saliva sample was collected at any point during the infant's hospitalization. We reviewed the infant and maternal charts for demographic information, medical diagnoses, and details of maternal substance abuse treatment and NAS outcome measures. Infants were assessed and treated according to comparable institutional NAS treatment protocols. All infants were scored using a modified Finnegan scale.(21) Infants with 3 consecutive scores 8 or 2 consecutive scores 10 were started on first-line opioid replacement therapy, which was neonatal morphine solution (0.5 – 1.0mg/kg/day) at the Tufts institutions and methadone (0.5 – 1.0mg/kg/day) at EMMC. Second-line therapy was of phenobarbital (Tufts) or clonazepam (EMMC). Infants were weaned from morphine, methadone, and clonazepam as inpatients and monitored for 48 hours prior to discharge home. Phenobarbital weaning was completed as an outpatient.

Laboratory Methods

DNA Isolation—Blood and saliva samples were sent to the Tufts Medical Center Clinical and Translational Research Center (CTRC) Core Laboratory for DNA isolation. Blood samples collected in PaxGene DNA tubes (Qiagen, Valencia, CA) were frozen within 14 days at –70°C until DNA isolation was performed. Salivary specimens were stored at room temperature before DNA extraction using the prepIT-L2P kit (DNA Genotek, Ottawa, ON, Canada). DNA was genotyped for the *OPRM1* 118A>G (rs1799971, dbSNP database) SNP using established TaqMan technology (assay C_8950074_1, Life Technologies, Grand Island NY).

Bisulfite DNA Conversion—Genomic DNA (300 ng) was treated with sodium bisulfite using the EZ DNA Methylation Gold Kit #D5005 (Zymo Research, Orange, CA). The bisulfite-treated DNA was eluted in 20 µl M-Elution Buffer. The Human Methylated & Non-Methylated DNA Control Set (Zymo Research Cat# D5014) was mixed to create DNA

with various percentages of methylation (0%, 25%, 50%, 75%, 100%) to monitor the efficiency of the bisulfite treatment.

OPRM1 CpG Methylation Analysis—Cord blood and saliva methylation levels were used as biomarkers for methylation levels occurring in the central nervous system. *OPRM1* methylation analysis was conducted according to methods published by Nielsen et al with minor modifications (17). Two CpG islands were located from 400 nucleotides upstream to 1000 nucleotides downstream of the transcription start site in the *OPRM1* promoter (Figure 1). The first CpG island is located from -97 to +27, labeled relative to the A of the ATG translation start site. We examined sixteen CpG dinucleotides located at nucleotide -93, -90, -80, -71, -60, -50, -32, -25, -18, -14, -10, +12, +23, +27, +53, and +84. The -18, -14, -10, +12 and +84 CpG sites are located at potential Sp1 transcription factor binding sites.

Primers for amplifying the upstream *OPRM1* CpG island were as follows: 1) primer A: 5'-TTTTTTTTTTGTTTTAGTTAGG-3'; 2) primer B: 5'-CAAATTACCATCTAAATAAA-3'; 3) primer C: 5'-TGTAAGAAATAGTAGGAGTTGTGGTAG-3'; and 4) primer D: 5'-AATAAAACAAATTAACCCAAAAACC-3'. The first amplification was performed with 1 µl bisulfite-treated DNA, 1 µM each of primers A and B, 250 µM each of dATP, dCTP, dGTP and TTP, 4 mM MgCl₂, 0.625 units of HotStarTaq *Plus* DNA Polymerase (Qiagen, Valencia, CA), and Qiagen PCR Buffer in a final volume of 50 µl. Amplification consisted of 5 min at 95°C, 40 cycles of 15 sec at 95°C, 15 sec at 52°C, and 30 sec at 72°C, followed by a final elongation step at 72°C for 7 min. A nested PCR reaction was performed using the same conditions as above with 1 µl of the initial PCR product and primers C and D. The nested amplification consisted of 5 min at 95°C, 40 cycles of 15 sec at 95°C, 15 sec at 58°C, and 30 sec at 72°C, followed by a final elongation step at 72°C for 7 min.

Direct sequencing of OPRM1 CpG—Preparation for sequencing was according to Nielsen et al with minor modifications.(17) Briefly, unincorporated nucleotides and primers were treated by mixing 5 µl of the nested PCR reaction mixture with 2 µl ExoSAP-IT (USB Corp., Cleveland, Ohio) followed by incubation at 37°C for 15 min and 80°C for 15 min. For an appropriate concentration for sequencing (5–10 ng/µl), 1 µl of the ExoSAP-IT-treated DNA was diluted 1:50 with water. For sequencing, 2 µl of the diluted ExoSAP-IT-treated DNA was added to 5 µM primer C or D in a final volume of 7.6 µl. Sequencing was performed on an ABI 3130 XL sequencer (Applied Biosystems).

Determination of Percent DNA Methylation—Trace files (.ab1) were analyzed using the ESME version 3.2.1 software from Epigenomics AG (Berlin, Germany). The percent methylation calls by the ESME were visually inspected using the associated electropherograms generated by the ESME software. Electropherograms were reviewed twice for accuracy. The promoter region was analyzed for predicted transcription factor binding sites using TESS: Transcription Element Search System, Patch 1.0 and AliBaba 2.1. (22–23)

Statistical Analyses

Our primary outcome measure for NAS severity was need for any NAS pharmacologic treatment, with secondary outcome measures of treatment with ≥ 2 medications (yes/no) and length of stay. Because infants treated with ≥ 2 medications represent the most severe phenotype of NAS, this factor was selected as a key outcome measure. Total opioid treatment days correlated strongly with length of stay ($r=0.92$; $P<0.001$), thus opioid treatment days are not reported separately. We selected candidate variables for adjusted analyses by comparing treated versus non-treated infants in bivariable analysis using chi-square (test of independence) and independent sample t-tests. Breastfeeding (yes/no) was defined as any amount of mother's milk consumed during the hospitalization as documented in the infant's medical chart. Then we averaged methylation levels across 16 CpG sites for each subject and tested the association of the subject level average (mean) with potential co-variables using independent sample t-tests.

We tested the association of NAS outcome measures with level of methylation for each of the 16 CpG sites within the *OPRM1* promoter region. Methylation levels were also compared among those infants who were treated with none, one, or two medications at each CpG site using ANOVA. Linear regression models were then created to adjust for co-variables associated with methylation levels or NAS outcomes with $P<0.05$ in bivariate analysis. Lastly, we applied the Benjamini and Hochberg method to account for the testing of 16 CpG sites in *OPRM1* for each of the NAS outcomes.(24)

Genotype frequencies were assessed for differences from the HapMap CEU database using the chi-square test (goodness of fit) and for Hardy-Weinberg equilibrium.(25) Lastly, we used independent sample t-test to compare methylation levels based on genotype in the *OPRM1* 118A>G SNP using a dominant genetic model (AA genotype vs AG/GG genotypes). Statistical analyses were performed with R programming (2010).

RESULTS

Of the 86 infants, 84 (98%) were Caucasian and 70 (81%) were ≥ 38 weeks gestational age. Fifty-five (64%) of the infants were exposed to maternal methadone [mean dose at delivery 106 mg (95% CI 81 – 124)] and 31 (36%) were exposed to buprenorphine [mean dose at delivery 16 mg (95% CI 13 – 19)]. Sixty-seven (78%) of the infants also had concurrent *in-utero* nicotine exposure; 10 (12%) benzodiazepines, and 4 (5%) selective serotonin reuptake inhibitors (SSRIs). Average length of stay for all infants was 22 days (95% CI, 19–26 days); and for treated infants, 32 days (95% CI, 28–35 days). Fifty-six (65%) of all infants were treated for NAS with 38% of these infants also treated adjunctively with phenobarbital ($n=16$) or clonazepam ($n=5$).

Demographic variables were compared between treated and non-treated infants as shown in Table. Medical co-morbidities and maternal medical factors did not differ between the infants. Breastfed infants had decreased length of stay (16 vs 27 days; $p<.001$) and decreased need for any medical treatment for NAS (63% of non-treated versus 34% of treated infants were breastfed, $p=.009$). There were no significant differences when comparing Tufts and the affiliated hospitals with EMMC for NAS outcome measures.

Maternal treatment and doses of these medications at delivery did not correlate with any NAS outcome measure.

DNA samples including 24 from cord blood and 62 from saliva. Methylation levels across the 16 CpG sites did differ between infant DNA sources with a higher mean level of methylation from cord blood compared with saliva samples [10.0% (95% CI 8.1 – 11.9) vs 6.7% (95% CI 5.5 – 7.9), $p=0.003$]. However, mean methylation levels did not differ based on DNA source at the -14, -10, and +84 CpG sites.

DNA Methylation Levels and Genotype

For the *OPRM1* 118A>G SNP (rs1799971), the genotype frequencies were: AA 0.71, AG 0.28, and GG 0.01, with corresponding allele frequencies of A = 0.85 and G = 0.15. Hardy-Weinberg equilibrium was not violated ($p=.42$) and there were no differences from the frequencies observed with the HapMap CEU population ($p>0.05$). There were no differences in methylation levels between genotypes at each of the 16 CpG sites.

DNA Methylation Levels and NAS Outcomes

Results for our primary outcome measure of need for any treatment for NAS by level of DNA methylation at each CpG site are shown in Figure 2. Methylation was increased at the -10 CpG in those infants receiving any treatment for NAS compared with non-treated infants in a model that adjusted for infant DNA source and breastfeeding [unadjusted difference 2.8% (3.0% vs 5.8%); adjusted difference $\delta=3.2\%$ (95% CI 0.3 – 6.0%), $p=0.03$]. After adjustment for multiple testing, results were no longer significant. No additional CpG sites were found to be associated with need for treatment of NAS.

The need for treatment with 2 medications for NAS by level of DNA methylation at each of the 16 CpG sites is shown in Figure 3. Methylation was increased at the -14 [unadjusted difference 5% (5.2 vs 10.2%); adjusted $\delta=4.9\%$ (95% CI 1.8 – 8.1%), $p=0.003$], -10 [unadjusted difference 5.0% (3.6 vs 8.6%); adjusted $\delta=5.0\%$ (95% CI 2.3–7.7, $p=0.0005$)], and +84 [unadjusted difference 3.3% (1.7 vs 5.0%); adjusted $\delta=3.5\%$ (95% CI 0.6 – 6.4%), $p=0.02$] CpG sites in those infants requiring 2 medications compared with those treated with 0 – 1 medication. Linear regression models were adjusted for infant DNA source and breastfeeding. Results for the -10 and -14 CpG sites remained significant after correction for multiple testing. These three CpG sites are located at putative Sp1 transcription factor binding sites (Figure 1).

Although results were not significant after adjustment for multiple testing, a positive correlation was found between level of methylation and length of stay ($r=0.27$, $p=0.03$) at the -10 CpG site. In a model that adjusted for DNA source and breastfeeding, each 1% increase in methylation level corresponded to a 0.8 day increase in length of stay (95% CI 0.1 – 1.5 days, $p=0.02$). No other correlations were found with length of stay for the other CpG sites. Methylation levels also increased with the number of medications required to treat NAS [3.0% vs 4.6% vs 6.9% for 0, 1, and 2 medications respectively; $p=0.05$] in unadjusted analysis for the -10 CpG. The mean level of methylation for each subject averaging all 16 CpG sites did not correspond with any NAS outcome measures.

DISCUSSION

Our results are consistent with the prior studies evaluating the importance of the *OPRM1* gene in opioid addiction and NAS. The common 118A>G rs1799971 SNP causes to an amino acid change resulting in a 3-fold increase in the binding affinity of the receptor with β -endorphin, altering the function of the hypothalamic-pituitary-adrenal axis, and vulnerability to addiction.(26–27) Data from animal models and *in vivo* studies indicate that the G allele is associated with a decrease in protein expression and gene expression.(28–29) We found that infants with NAS who carried at least one copy of this minor G allele had a shorter length of stay and were less likely to receive any treatment for NAS compared with infants who were homozygotes for the A allele, suggesting improved tolerance to the process of opioid withdrawal.(6)

In the present study, opioid exposed infants with a more severe phenotype of NAS had an increase in DNA methylation of three CpG sites in the *OPRM1* promoter region. An increase in DNA methylation levels at the –14, –10, and +84 CpG sites were found in infants who required two or more medications to control withdrawal symptoms. The –14, –10, and +84 CpG sites are located at putative Sp1 transcription factor binding sites.(23) With an increased level of DNA methylation, Sp1 sites have a decreased binding affinity for their transcription factors which may lead to a decrease in gene expression.(30–31) In this case, infants with hypermethylation at these specific CpG sites may have down-regulated *OPRM1* gene expression leading to reduced levels of the mu-opioid receptor. This in turn may lead to an increased need for opioid medications to control NAS symptoms. Our results do not show that the opioids caused the hypermethylation at the three CpG sites, but that the hypermethylation of the *OPRM1* promoter region may influence NAS outcome measures. The impact of changes in methylation should be evaluated in future studies for correlations with the level of the mu-opioid receptor, as well as methylation levels in the mothers. The changes in methylation levels before and after treatment should also be evaluated to determine if opioid replacement alters any epigenetic changes in newborns with NAS.

Prior studies have also demonstrated that an increase in *OPRM1* promoter methylation is associated with a decrease in protein expression of the mu-opioid receptor.(15) Nielsen found increased levels of methylation at the –18 and +84 CpG sites in the *OPRM1* promoter, sites of putative Sp1 transcription factor binding, in lymphocytes of prior heroin addicts compared with controls.(17) They also found that methylation levels differed depending on ethnicity in former heroin addicts, with an increased level of methylation at the –14 site noted in Hispanics.(18) Chorbov and associates found hypermethylation at seven *OPRM1* CpG sites in leukocyte DNA of male opioid addicts compared with controls.(16) Similar changes at the –10 site were measured in European Americans with alcohol dependence compared with controls.(32) Transgenerational effects of chronic opioid exposure have been demonstrated in animal models, suggesting that epigenetic changes in the mother due to opioid addiction may be passed on to her infant altering sensitivity to narcotics and the process of withdrawal.(33–34) The heritability of these changes is also supported by a study that evaluated sperm-derived DNA from opioid addicts, finding that methylation was increased at one CpG site within *OPRM1*.(16)

This study has a number of limitations. First, though the same NAS scoring system was used at all study centers, an intra-observer reliability program was not established between sites. Although the Finnegan scoring system is the gold standard, it remains subjective with the possibility for differences between institutions and individual providers. This study is also limited by a small sample size with the need for correction for multiple statistical comparisons, limiting our statistical power. Our sample size likely accounts for why more significant differences were not seen between treated and untreated infants. However, higher methylation levels were seen in those infants requiring 2 medications, representing the most severe NAS phenotype. The generalizability of our results is also limited by lack of ethnic variability as methylation levels vary depending on ethnic background.(18) However, our results are consistent with prior studies in opioid dependent Caucasian adults.(17)

The timing of the DNA samples varied with some samples collected prior to initiation and some during treatment with opioid medications for NAS, which may have influenced methylation levels. The source of DNA accounted for some of the variation in methylation, with differences seen in mean methylation levels between cord blood and saliva samples. Cord blood and saliva methylation levels can be considered as biomarkers for *OPRM1* methylation in the central nervous system.(35) Although DNA quantity and quality are typically higher from cord blood compared with saliva or buccal cells, it is often difficult to obtain cord blood at the time of delivery from all subjects.(36) An increasing number of epigenetic studies have utilized saliva as a DNA source as it is readily available, can be followed serially, and is non-invasive.(35,37) DNA source was adjusted for in all of our multivariate models and results remained unchanged when DNA source was included in the models.

The identification of key genetic factors through early non-invasive testing that correlate with NAS severity can lead to future individualized treatment regimens and improved care for these infants. Infants with high risk genetic profiles could be treated more aggressively from birth, and those with low risk profiles could potentially be discharged home from the hospital earlier. Identifying similar genetic markers in the mothers and placentas also represent an opportunity for earlier identification of high-risk infants.

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Figure 1. *OPRM1* Promoter Region

The *OPRM1* gene promoter region is shown with the two CpG islands boxed in and CpG dinucleotides indicated as |. The major transcription start site is indicated by the arrow, located -253 upstream of the ATG translation start site. The sequence of the amplified CpG island is also shown with the 16 CpG sites analyzed for cytosine methylation (bold) and their position relative to the ATG translation start site (underlined) indicated. Three putative Sp1 transcription factor binding sites are boxed.

Figure 2.

Percent methylation of 16 CpG sites within the *OPRM1* promoter in opioid-exposed infants who were treated (n=65) versus non-treated (n=21) for NAS. CpG sites in Sp1 transcription factor binding sites are indicated. * p<0.05 in bivariable analyses.

Figure 3.

Percent methylation of 16 CpG sites within the *OPRM1* promoter in opioid-exposed infants treated with ≥ 2 medications (n=21) versus < 2 medications (n=65) for NAS. CpG sites in Sp1 transcription factor binding sites are indicated. * p<0.05 in bivariable analyses.

Table 1

Demographics in infants treated versus not treated for NAS

Variable	Non-Treated Infants n=30 No.(%)	Treated Infants n=56 No.(%)	P-Value
GA 38 weeks	25 (83%)	45 (80%)	0.74 ^c
Birth Weight (kg) mean (95% CI)	3.2 (3.0–3.4)	3.2 (3.0–3.3)	0.89 ^d
Maternal Opioid Substitute			
Methadone	17 (57%)	38 (68%)	0.30 ^c
Buprenorphine	13 (43%)	18 (32%)	
Methadone Dose (mg)^a mean (95% CI)	100 (76–124)	109 (72–146)	0.57 ^d
Buprenorphine Dose (mg)^a mean (95% CI)	16 (11–20)	16 (13–19)	0.96 ^d
Breastfed^b	19 (63%)	19 (34%)	0.009^c
Concurrent Exposure			
Cigarette Smoking	22 (73%)	45 (80%)	0.45 ^c
Benzodiazepines	3 (10%)	7 (13%)	0.69 ^c
SSRIs	2 (7%)	2 (4%)	0.41 ^c
Tufts & Affiliates	16 (53%)	35 (63%)	0.41 ^c
EMMC	14 (47%)	21 (38%)	

Abbreviations: GA = gestational age; mg = milligrams; kg = kilograms; SSRI = selective serotonin re-uptake inhibitor; EMMC = Eastern Maine Medical Center

^a Mean daily dose at delivery;

^b breastfed to any extent;

^c P-value calculated by independent sample t-test;

^d P-value calculated by chi-square test of independence