DNA Methylation of Neurodevelopmental, Stress-Response, Reward and Opioid Pathway Genes in Infants with Neonatal Opioid Exposure: Exploration of Sex-Specific Associations

By

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Introduction

Public Health Significance

The opioid epidemic has profoundly impacted maternal and infant health in the United States, with prenatal opioid exposure recognized as a major public health concern. A 2019 self-reported survey found that approximately 7% of pregnant women reported prescription opioid use during pregnancy, with nearly 20% reporting misuse (Ko, 2020). Correspondingly, maternal opioid-related diagnoses at delivery increased by 131% between 2010 and 2017, while diagnoses of Neonatal Opioid Withdrawal Syndrome (NOWS), formerly known as Neonatal Abstinence Syndrome (NAS), increased by 82%, from 4.0 to 7.3 per 1000 hospital births (Hirai et al., 2021). These rising rates underscore the growing clinical and societal burden of opioid exposure during pregnancy.

NOWS is a withdrawal syndrome that occurs following prenatal opioid exposure. Symptoms typically emerge within 72 hours of birth and reflect neurologic, autonomic, and gastrointestinal dysregulation (Jansson et al., 2009). Clinical manifestations include excessive crying, difficulty sleeping, tremors, poor feeding, diarrhea, vomiting, increased muscle tone, seizures, fever, and irritability (Hudak et al., 2012). These symptoms can significantly disrupt early neonatal adaptation and often require prolonged hospitalization.

Management of NOWS is tiered according to symptom severity. American Academy of Pediatrics (AAP) guidelines for the management of NOWS emphasize first-line treatment consisting of non-pharmacological interventions such as swaddling, skin-to-skin contact, pacifier use, and rooming-in with the mother (Patrick et al., 2020). For infants with more severe withdrawal, pharmacologic therapy is initiated with an opiate, such as morphine, methadone or buprenorphine to reduce withdrawal symptoms and support feeding, weight gain, and physiologic stability. Adjunct medications such as phenobarbital or clonidine may also be administered to improve neurobehavioral regulation and reduce total opioid exposure (Agthe et al, 2009; Coyle et al., 2002, 2018). As NOWS prevalence increases, there is a growing need to better understand the factors that contribute to withdrawal risk and severity to inform and optimize treatment decisions.

Heterogeneity in NOWS presentation.

Although numerous maternal, infant, and environmental factors have been associated with NOWS risk, findings remain inconsistent. Increased gestational age and higher birth weight have been associated with a greater risk of NOWS, whereas breastfeeding and rooming-in have been associated with reduced risk (Abrahams et al., 2007; Cleary et al., 2012; Kaltenbach et al., 2012; Liu et al., 2010; MacMillan et al., 2018; Ruwanpathirana et al., 2015; Seligman et al., 2008; Welle-Strand et al., 2013). Although this may appear counterintuitive, older and larger infants may have experienced longer in utero opioid exposure and exhibit more mature and clinically observable withdrawal phenotypes, increasing the likelihood of detection and pharmacological treatment (Doberczak et al., 1991; Seligman et al., 2008). This difference may reflect not just biology, but also differences in how withdrawal is expressed and detected across gestational ages. The complexity of diagnosis, particularly in preterm infants, is further increased by the lack of validation of scoring tools such as the Finnegan in preterm infant populations, particularly given that prenatal opioid exposure is associated with a higher risk of preterm labor (Amiri & Nair, 2022; Graeve et al., 2022). In addition to these diagnostic challenges, polypharmacy has been consistently associated with increased severity of NOWS, particularly with co-exposure to opioids and benzodiazepines or other psychotropic medications (Sanlorenzo et al., 2019; Huybrechts et al., 2017; Bakhireva et al., 2022). In contrast, evidence remains mixed regarding associations with opioid dose and infant sex (Kelty & Preen, 2019). Additional factors such as maternal drug history, maternal metabolism, placental transfer of opioids, infant metabolism, and genetic variability may further influence withdrawal expression (Hudak et al., 2012). Critically, not all infants exposed to opioids prenatally develop NOWS, and the mechanisms that determine which infants will experience clinically significant withdrawal remain poorly understood (Kelty & Preen, 2019).

Despite similar prenatal opioid exposures, infants demonstrate marked heterogeneity in NOWS presentation, reflecting the complexity described. Variability is observed in the timing of symptom onset, degree of symptom severity, need for pharmacological treatment, and duration of hospitalization (Coyle et al., 2018). Measures of NOWS severity vary across studies and clinical settings. Common outcome variables include length of hospital stay, duration, dose, and type of pharmacological treatment, including need for adjunctive medications, maximum withdrawal

scores, and atypical neurobehavioral profiles (Flannery et al., 2020). This variability complicates comparisons across studies and highlights the absence of standardized markers of severity.

Compounding this heterogeneity is the subjectivity in diagnosing NOWS resulting in treatment thresholds that differ across institutions and regions, reflecting variation in clinical protocols (Johnson et al., 2025). The most commonly used assessment tool for NOWS is the FNAST and its variants, including the modified version, which assigns a cumulative score based on 21 observed withdrawal-related symptoms such as cry characteristics, sleep disturbance, tremors, and gastrointestinal dysfunction, with infants typically assessed every 2 to 4 hours (Finnegan, Connaughton, et al., 1975; Finnegan, Kron, et al., 1975; Hudak et al., 2012; Maguire et al., 2013). The Neonatal Withdrawal Inventory (NWI) provides a shorter, 8-item checklist intended for more rapid assessment (Maguire et al., 2013; Zahorodny et al., 1998). More recently, the Eat, Sleep, Console (ESC) approach has shifted focus toward infant functional capacity, evaluating the ability to feed, sleep, and be consoled rather than quantifying individual symptoms (Schiff & Grossman, 2019). Because these tools differ in structure and scoring criteria, reported NOWS rates and treatment decisions may vary depending on the assessment method used (Young et al., 2023). Importantly, all these approaches rely on observer-rated symptom scales that detect withdrawal after it has emerged. As a result, diagnosis and treatment decisions remain subjective, making a consensus regarding treatment difficult.

Collectively, these findings highlight a central gap in the field. While clinical and demographic correlates of NOWS have been described, there are currently no objective biological predictors capable of explaining why some infants exposed to opioids develop severe withdrawal while others remain minimally affected. Identifying the biological mechanisms underlying this heterogeneity is essential for improving risk stratification and advancing more precise, proactive management of NOWS.

Epigenetic Mechanisms as a Pathway for Heterogeneity

Genetic variation has been investigated as a contributor to NOWS severity. *OPRM1,* which encodes the mu-opioid receptor, has been one of the most extensively studied genes given its direct involvement in opioid signaling and addiction (Wachman & Farrer, 2019). Specific alleles and single-nucleotide polymorphisms (SNPs) within *OPRM1* have been associated with decreased

NOWS severity but increased risk of opioid addiction later in life (Wachman et al., 2013). Additional genes examined in relation to NOWS include opioid receptor and peptide genes such as *OPRK1, OPRD1*, and *PNOC*, as well as stress response and dopaminergic pathway genes, including *COMT* and *DRD2* (Levran et al., 2012; Oei et al., 2012; Wachman et al., 2013, 2015). These findings suggest that genetic variation may partially explain differences in withdrawal expression among infants exposed to opioids.

Beyond genetic variation, epigenetic differences between infants represent an additional pathway that may contribute to heterogeneity in NOWS presentation. While genetic variation reflects differences in DNA sequence, epigenetics refers to regulatory processes that alter gene expression without modifying the underlying genetic code. These processes include DNA methylation (DNAm), histone modifications, and non-coding RNAs. Among these mechanisms, DNAm is the most extensively studied. DNAm primarily occurs at CpG sites, where a cytosine nucleotide is followed by a guanine nucleotide linked by a phosphate bond. CpG sites are often clustered in CpG islands upstream of transcription start sites, a region known as the promoter region. Increased promoter methylation is typically associated with reduced gene expression (Bird, 1986; Felsenfeld, 2014). Because prenatal exposures occur during sensitive developmental windows, they may alter DNAm patterns in ways that influence gene expression and subsequent infant outcomes.

The in-utero period represents a critical developmental window during which environmental exposures can shape epigenetic regulation and influence long-term phenotypic outcomes (Marsit, 2015). DNAm plays a central role in early developmental programming, as widespread epigenetic reorganization during early embryonic development helps regulate activation of the embryonic genome and establish patterns of gene expression necessary for normal growth and differentiation. During this time, DNAm marks are dynamically established and refined, regulating gene expression by influencing whether genes are activated or silenced and laying the foundation for later tissue-specific gene regulation and long-term biologic function. Because these patterns are being actively set during fetal development, they are particularly sensitive to maternal environmental conditions. Adverse intrauterine exposures have been shown to disrupt the early embryonic epigenome (Breton-Larrivée & Elder, 2019). More broadly, growing evidence links prenatal exposure to environmental toxins, including tobacco smoke, air pollution, and drug use, to altered DNA methylation patterns in offspring (Knopik et al., 2019; Yang & Schwartz, 2012).

Within this framework, fetal epigenetic programming has been proposed as an adaptive mechanism through which a developing organism calibrates gene expression in anticipation of the postnatal environment, although such adaptations may also confer vulnerability when environmental demands are mismatched (Marsit, 2015).

Emerging evidence suggests that prenatal opioid exposure may similarly alter epigenetic regulation. Previous studies have demonstrated that prenatal opioid exposure is associated with changes in DNAm patterns within opioid-related genes (McLaughlin et al., 2017). Because a substantial proportion of DNAm patterning is established during pregnancy, these marks are particularly vulnerable to environmental and drug exposures during this period. To date, most epigenetic studies of NOWS have focused on single or candidate genes, particularly *OPRM1*. Increased methylation at specific *OPRM1* CpG sites has been associated with increased severity of NOWS, requiring primary and adjunctive pharmacological treatment (Wachman et al., 2014, 2018). Additional studies have reported increased DNAm in *OPRM1*, as well as in *ABCB1*, a drug transport gene, and *CYP2D6*, a cytochrome P450 enzyme involved in opioid metabolism, among newborns exposed to opioids compared to opioid naïve infants (McLaughlin et al., 2017). Collectively, these findings suggest that epigenetic variation, particularly within opioid signaling pathways, may represent a biologically plausible mechanism contributing to heterogeneity in NOWS severity.

Sex differences

Infant sex may represent an additional biological contributor to heterogeneity in NOWS severity, as male and female fetuses demonstrate well-established differences in developmental trajectories and responses to prenatal stress. Epidemiologic studies consistently show that more males than females are born preterm (Cooperstock & Campbell, 1996) and that males experience poorer neonatal and infant health outcomes (Peacock et al., 2012). Environmental stressors during early life also appear to disproportionately affect males, resulting in greater vulnerability under adverse conditions (Wells, 2000). These sexually dimorphic responses are thought to be programmed early in gestation and mediated, in part, by differences in placental function and stress responsiveness (Clifton, 2010). Together, these findings suggest that biological sex shapes fetal adaptation to

prenatal environments and may influence susceptibility to conditions arising from intrauterine stress.

Building on these developmental differences, emerging evidence suggests that sex-specific biological variation may translate into clinically meaningful differences in NOWS presentation and long-term outcomes. In humans, male infants exhibit more severe withdrawal symptoms and receive pharmacological treatment more frequently than females (Jansson et al., 2007; Charles et al., 2017). Beyond the neonatal period, sex-specific vulnerability appears to persist, with studies demonstrating lower cognitive functioning among boys with prenatal opioid exposure compared to unexposed male controls, whereas comparable differences are not consistently observed among girls (Skumlien et al., 2020). Similar patterns have been reported in the context of prenatal polypharmacy exposure, including opioids, where boys show more persistent cognitive deficits over time when compared to male unexposed controls (Nygaard et al., 2015; Moe & Slinning, 2001). Collectively, these data indicate that male infants may be particularly susceptible to the effects of prenatal opioid exposure.

Sex-specific differences in molecular responses to prenatal opioid exposure have been observed. Gene expression patterns differ between males and females across inflammatory, dopaminergic, and metabolic pathways. Opioid exposed females show higher expression of inflammatory mediators compared to males, including differences in *CCL2* and *CXCL1*, particularly among infants requiring treatment (Yen et al., 2023). In contrast, opioid exposed males requiring treatment had higher expression of *DRD2* relative to females (Yen et al., 2023). Prior work similarly reported increased *DRD2* expression in male newborns with NOWS compared with female newborns with NOWS, along with sex-specific differences in appetite-related genes, including the leptin receptor and *NPY2R* (Yen et al., 2019). Together, these findings support the possibility that sex-specific biological mechanisms, including differential epigenetic regulation, contribute to variability in NOWS severity and developmental trajectories.

Gaps in current research.

Although human genetic studies have identified multiple single-nucleotide polymorphisms associated with NOWS-related outcomes across opioid receptor and stress-response genes, research on the epigenetics of NOWS remains comparatively limited. Most DNAm studies in

NOWS have focused primarily on *OPRM1*, rather than evaluating regulatory-region methylation across multiple biologically relevant pathways (Wachman & Farrer, 2019). This narrow emphasis constrains mechanistic understanding, as NOWS reflects dysregulation across stress physiology, neurobehavioral regulation, feeding, and reward systems rather than isolated alterations in opioid receptor signaling. While epigenetic variation in *OPRM1* has been linked to withdrawal (Camerota et al., 2022; Wachman et al., 2014, 2018), comparatively little research has examined DNAm in genes involved in biological stress response, including *NR3C1*, *AVP, SLC6A4, OXTR,* and *FKBP5,* neurodevelopmental processes such as *BDNF*, or dopaminergic signaling pathways including *DRD2* and *DRD4*. Prenatal stress and adversity have been associated with DNAm variation in *NR3C1* in human studies (Hompes et al., 2013; Mulligan et al., 2012), and early-life stress has been linked to persistent epigenetic changes in *AVP* regulatory regions in experimental models (Murgatroyd et al., 2009). Altered methylation in *OXTR* and *SLC6A4* has been associated with prenatal stress, substance exposure and early infant temperament (Baptista et al., 2021; Provenzi et al., 2021; Unternaehrer et al., 2016), and *BDNF* methylation has been associated with prenatal and neonatal stress exposure (Kertes et al., 2017). In parallel, genetic variation in *FKBP5, DRD2,* and *DRD4* has been associated with heroin addiction and opioid dependence (Chen et al., 2011; Levran et al., 2014), underscoring the relevance of these pathways to opioid-related phenotypes. Collectively, these findings suggest that regulatory-region DNAm in stress-related, neurodevelopmental, and dopaminergic genes may plausibly influence withdrawal expression. However, these pathways have not been systematically examined in relation to NOWS severity within a single framework.

In addition, despite evidence that NOWS outcomes differ by infant sex (Charles et al., 2017; Jansson et al., 2007), few studies have tested whether epigenetic associations with NOWS severity are sex-specific. This represents an important limitation, given established sex differences in fetal adaptation to prenatal stress and in molecular responses to opioid exposure (Clifton, 2010; Yen et al., 2019, 2023). Clarifying sex-specific epigenetic pathways may improve mechanistic insight into withdrawal expression and support the identification of early biomarkers predictive of clinically significant NOWS, thus advancing understanding of NOWS heterogeneity and informing earlier risk stratification in newborns exposed to opioids.

Accordingly, the objective of the present study was to investigate associations between DNAm in regulatory regions of neurodevelopmental (*BDNF*), stress-response (*AVP, NR3C1, OXTR, FKBP5, SLC6A4*), dopaminergic (*DRD2, DRD4*), and opioid signaling (*OPRM1*) pathway genes and the need for pharmacological treatment among newborns exposed to opioids. We also investigated whether the associations between DNAm and need for NOWS treatment differed by infant sex. We hypothesized that DNAm at regulatory CpG sites within these genes would differ between infants who required pharmacological treatment and those who did not, and that these associations would vary by infant sex.

Methods

Participants

Participants in the Child and Family Study (CAFS) were part of a prospective, multisite investigation aimed at identifying novel clinical indicators of NOWS. Recruitment of 235 mother-infant dyads with prenatal exposure to opioids took place at Women and Infants Hospital of Rhode Island (WIH) and the University of Utah Hospital between 2019 and 2025. Mothers were approached for consent if prenatal opioid exposure was identified either during pregnancy or delivery as indicated by maternal medical record, a positive maternal urine toxicology during pregnancy or at time of hospital admission, or a positive infant toxicology screen as seen in the umbilical cord or urine after birth. Exclusion criteria included congenital anomalies, genetic syndromes, metabolic disturbances, sepsis, asphyxia, seizures, respiratory failure, gestational age < 33 weeks, inability to take oral medication, inability to provide informed consent, and inability to carry out a newborn neurobehavioral exam. The Institutional Review Board approved procedures for each study site, and written consent was obtained from all participants.

Need for pharmacological treatment

Information regarding NOWS symptoms and need for pharmacological treatment was obtained from the infant's medical record. Assessment of withdrawal severity and criteria for initiating pharmacological treatment followed site-specific clinical protocols. At WIH, NOWS symptoms were evaluated using the FNAST. Pharmacological treatment was initiated following either three consecutive FNAST scores greater than 7 or two consecutive scores greater than 11. At the University of Utah Hospital, withdrawal symptoms were assessed using the NWI and the ESC approach. The NWI is an empirically derived eight-item instrument adapted from the FNAST, with treatment initiated when an infant received one or more scores of 8 or higher. The ESC method evaluates functional indicators of withdrawal, including the infant's ability to feed appropriately for age, sleep undisturbed for at least one hour between care periods, and be consoled within 10 minutes; failure to meet any of these criteria could prompt initiation of pharmacological treatment. Pharmacological treatment for NOWS at both sites primarily consisted of oral morphine, with phenobarbital administered as additional therapy for infants who exceeded a

predetermined opioid dose threshold. Because assessment tools and treatment thresholds differed across sites, study site was included as a covariate in all statistical analyses.

Sequencing

DNA was extracted from buccal swabs collected from newborns shortly after birth, before initiating pharmacological treatment. Quantitative DNAm was analyzed as described previously (Padbury et al., 2022). In summary, QIAamp DNA Mini Kits (Qiagen, Inc.) were used to extract genomic DNA from saliva samples by following the manufacturer's protocols. Pyrosequencing (Qiagen Inc.) was then used on the purified DNA to carry out quantitative methylation analysis. The EZ DNA Methylation Kit (Zymo Research) was used to bisulfite-modify the DNA samples (1µg). The PCR amplified products from the bisulfite-modified DNA were pyrosequenced on a Qiagen Q48 pyrosequencing platform using assay reagents from EpigenDx for CpG sites within promoter regions of neurodevelopmental (*BDNF*), stress-response (*AVP, NR3C1, OXTR, FKBP5, SLC6A4*), dopaminergic (*DRD2, DRD4*), and opioid signaling (*OPRM1*) pathway genes. The performance characteristics of the assay were evaluated using a dilution series of fully methylated reference DNA into fully unmethylated reference DNA. DNAm values are reported as percent methylation at each CpG site. Standard assay quality control procedures were applied, and CpG sites with unreliable signal were excluded prior to analysis.

Statistical Analysis

DNAm data were analyzed at CpG sites located within the 5′ upstream and untranslated (UTR) regulatory regions of genes of interest (*BDNF, AVP, NR3C1, OXTR, FKBP5, SLC6A4, DRD2, DRD4*, and *OPRM1*). DNAm within these regions may influence gene transcription, as methylation near transcription start sites can alter promoter activity. DNAm at individual CpG sites was examined to identify associations with the need for pharmacological treatment. Analyses were conducted in a stepwise manner. Robust linear regression and non-parametric tests were selected due to the non-normal distribution commonly observed in DNAm data. First, unadjusted group differences between infants who required pharmacological treatment and those who did not were assessed using Wilcoxon rank-sum tests. Robust linear regression models (RLMs) were then used to examine associations between DNAm at each CpG site and subsequent need for pharmacological treatment, controlling for infant sex and study site. These covariates were chosen

due to known sex differences in neurodevelopment and differences in clinical management of NOWS across sites. To evaluate whether associations between DNAm and need for pharmacological treatment differed by sex, additional RLMs were fitted that included a sex-by-treatment interaction term, with study site retained as a covariate. To correct for multiple comparisons, *p*-values from the regression models were adjusted using the false discovery rate (FDR) procedure within each gene. Statistical significance was defined as FDR-adjusted $p < .10$. For CpG sites with a significant Sex x Treatment interaction term, estimated marginal means (EMMs) were calculated to quantify sex-specific differences in DNAm by treatment status. All analyses were conducted in R (version 4.5.2). RLMs were fit using the MASS package, and estimated marginal means were calculated using the emmeans package. Analyses were conducted using complete case data.

Results

Descriptive Statistics

Of the 235 enrolled mother–infant dyads, 181 had DNAm data and were included in the analyses. A comparison of maternal and infant characteristics between included and excluded dyads found no significant differences between groups except for maternal bipolar disorder diagnosis, which was more common in the included dyads (26.4% vs 9.4%, $p = .02$; **Supplemental Table 1**).

Table 1 presents descriptive maternal and infant characteristics for the 181 mother–infant dyads included in the sample, separated by pharmacological treatment status and infant sex. Overall, 76 infants (42%) required pharmacological treatment for NOWS, and 89 infants (49%) were male. Need for treatment did not vary by infant sex (42.7% in males, 41.3% in females, $p = 0.88$). Among maternal characteristics, no significant differences by treatment status or sex were observed for sociodemographic, substance use, or medical variables (all $p > .05$). One exception was that mothers of infants who required pharmacological treatment were more likely to have a diagnosis of bipolar disorder compared to mothers of infants who did not require pharmacological treatment (36% vs. 19%, $p = .02$).

Among the infant characteristics, gestational age, birth weight, head circumference, and birth length did not differ significantly by treatment status (all $p > .05$). However, infants who required pharmacological treatment for NOWS were more likely to have Social Services involvement (61% vs. 38%, $p = .004$) and were less likely to be breastfed (46% vs. 70%, $p = .003$). As expected, infants who required pharmacological treatment had higher maximum FNAST scores compared to those who did not ($M = 12.34$ vs. 7.80, $p < .001$).

Associations Between DNAm and Need for Pharmacological Treatment

Complete CpG-level results for all candidate genes are provided in the **supplemental file**, including effect estimates (β), nominal *p*-values, and false discovery rate–adjusted *p*-values for associations between DNAm and need for pharmacological treatment. At the nominal significance threshold ($p < .05$), differences in DNAm associated with the need for pharmacological treatment were observed across several genes. Higher DNAm was observed in infants who later required

pharmacological treatment relative to those who did not at one CpG site within *NR3C1* (distance from TSS = 196), one CpG site within *BDNF* (distance from TSS = -2021), and four CpG sites within *DRD4* (distance from TSS = -351, -338, -303, -301). In contrast, lower DNAm was observed among infants who later required pharmacological treatment relative to those who did not at one CpG site within *OPRM1* (distance from TSS = 226), and one CpG site within *DRD4* (distance from TSS = -333). No nominally significant main effects of treatment were observed within *AVP* or *SLC6A4.*

After FDR adjustment, *DRD4* was the only gene to retain statistically significant associations with need for pharmacological treatment. CpG-level results for all interrogated sites within the *DRD4* 5′ regulatory region are presented in **Table 2**, which includes both nominal and FDR-adjusted findings. Mean DNAm values presented in Table 2 are unadjusted, whereas β coefficients reflect adjusted estimates from regression models controlling for covariates. Four CpG sites demonstrated higher DNAm in infants who later required pharmacological treatment relative to those that did not (β = 0.47, distance from TSS = -351; β = 0.31, distance from TSS = -338; β = 0.43, distance from TSS = -303; β = 0.87, distance from TSS = -301), while one site showed significantly lower DNAm in infants who later received pharmacological treatment (β = -0.27, distance from TSS = -333; **Table 2**). **Figure 1** illustrates the distribution of DNAm levels at individual *DRD4* CpG sites by pharmacological treatment status.

Sex-Specific Associations Between DNAm and Treatment Status

At the nominal significance threshold ($p < .05$), Sex × Treatment interaction effects on DNAm were observed across several candidate genes within the 5′ regulatory regions (**Supplemental CSV**). *AVP* (distance from TSS = -6014) exhibited one CpG site with a nominal interaction effect, *NR3C1* (distance from TSS = 220, 267) exhibited two CpG sites, *BDNF* (distance from TSS = -2021, -2010) and *DRD4* (distance from TSS = -362, -358) each exhibited two CpG sites, and *DRD2* (distance from TSS = 285) exhibited one CpG site. No nominal Sex × Treatment interaction effects were observed within *FKBP5, SLC6A4, OXTR,* or *OPRM1*.

After FDR correction, statistically significant Sex × Treatment interactions were identified at two CpG sites: one within the *DRD4* gene and one within the *AVP* gene. These interaction terms indicate that the association between treatment status and DNAm differs by infant sex.

To interpret these interactions, EMMs were examined to characterize sex-specific patterns of DNAm by treatment status. For the *DRD4* CpG located -362 from TSS, we observed significantly higher DNAm among male infants who required pharmacological treatment relative to those that did not ($p < .05$). In contrast, there was no significant difference in DNAm by treatment status for female infants at this site (**Figure 2A**).

In contrast, the significant interaction observed at the *AVP* CpG located -6014 from the TSS showed a different pattern, with treatment-associated differences primarily observed among female infants. Specifically, female infants requiring pharmacological treatment exhibited lower DNAm compared to those who did not require treatment, although this within-group difference did not reach statistical significance ($p = .06$). No corresponding differences in DNAm by treatment status were observed among male infants **(Figure 2B)**.

Discussion

In this study, we investigated whether DNAm in regulatory regions of genes involved in stress signaling, neurodevelopment, dopaminergic reward pathways, and opioid receptor signaling was associated with need for pharmacological treatment among newborn infants with prenatal opioid exposure, and whether those relationships differed by infant sex. Our results showed that differential DNAm of several CpG sites within the regulatory region of *DRD4* was associated with need for pharmacological treatment for NOWS. In addition, we observed significant sex-specific associations with need for pharmacological treatment for CpG sites within *DRD4* and *AVP*. These findings suggest that epigenetic variation in dopaminergic and stress response pathways may contribute to variability in withdrawal severity among infants exposed prenatally to opioids and that these relationships may differ between male and female newborns.

DRD4 encodes the dopamine D4 receptor, which mediates dopaminergic signaling pathways implicated in reward processing, behavioral regulation, and feeding behavior. Dopaminergic pathways are known to be altered by opioid exposure and are also implicated in the neurobehavioral manifestations of withdrawal (Botticelli et al., 2020; Chen et al., 2011; Kocherlakota, 2014). Prior genetic studies have linked variation in dopaminergic receptors to substance use disorders and opioid dependence, including polymorphisms in *DRD2* and *DRD4* (Chen et al., 2011). In the context of neonatal withdrawal, altered methylation within the regulatory region of *DRD4* may influence receptor expression and downstream dopaminergic signaling. In our study, four CpG sites demonstrated greater DNAm in infants who required treatment, while one site showed lower methylation. Because increased methylation within promoter regions is generally associated with reduced gene expression, whereas decreased methylation may be associated with increased gene expression (Jones, 2012), these findings may suggest that altered DRD4 expression could contribute to behavioral regulation and feeding patterns that are commonly observed in infants with NOWS (Botticelli et al., 2020; Kocherlakota, 2014). While the functional consequences of the specific CpG sites examined in our study remain unclear, these findings suggest that dopaminergic regulatory mechanisms warrant further investigation in relation to withdrawal severity.

Although most previous work has focused on genetic variation rather than epigenetic regulation, emerging evidence suggests that methylation of *DRD4* can influence behavioral outcomes. For example, increased methylation of *DRD4* has been associated with greater cognitive and attentional deficits in children with attention-deficit/hyperactivity disorder, indicating that epigenetic regulation of this gene may influence behavioral and regulatory processes (Dadds et al., 2016). Prenatal opioid exposure has also been associated with increased risk of attention deficit hyperactivity disorder and related attentional difficulties in childhood (Schwartz et al., 2021). Given the established role of *DRD4* in attentional regulation and behavioral control, epigenetic variation in this gene may represent one pathway linking NOWS to later neurobehavioral outcomes.

Although the remaining candidate genes did not demonstrate associations with the need for treatment after FDR correction, several showed nominal associations consistent with prior research linking prenatal stress exposures to epigenetic regulation of stress pathways. However, these findings should be interpreted with caution, as they did not remain statistically significant after correction for multiple comparisons. In our study, variation in methylation across genes involved in HPA axis signaling (e.g., *NR3C1* and *OXTR*) and neurodevelopment (e.g., *BDNF*) suggested potential, although modest, relationships with need for pharmacological treatment. Previous work has shown that prenatal stress and maternal anxiety are associated with increased methylation within regulatory regions of *NR3C1*, which encodes the glucocorticoid receptor and plays a central role in HPA axis feedback (Hompes et al., 2013; Mulligan et al., 2012). Evidence from infant populations also suggests that methylation changes in *BDNF* are associated with prenatal and early neonatal stress exposures (Kertes et al., 2017). While these pathways are biologically relevant to stress regulation and neurodevelopment, the current study does not provide evidence of statistically significant associations between these genes and treatment need. These findings highlight the importance of further investigation in larger samples and more comprehensive epigenetic analyses.

In contrast to several previous candidate gene studies, this study did not observe a significant association between methylation of *OPRM1* and NOWS treatment status after FDR correction. Elevated methylation of *OPRM1* had previously been linked to both need for pharmacological treatment for NOWS and NOWS severity, including the length of hospital stay and need for multiple medications (Wachman et al., 2014, 2018). Other studies have also found that

pharmacological treatment for NOWS is associated with decreases in *OPRM1* DNAm and subsequent improvement in neonatal neurobehavior (Camerota et al., 2022). Differences in study design, sample size, tissue source, or the specific CpG sites examined may contribute to variation across findings. Despite our results not replicating earlier associations, they suggest that variability in NOWS may involve regulatory mechanisms across multiple biological pathways rather than opioid receptor signaling alone.

To our knowledge, this is the first study to examine sex-specific differences in epigenetic regulation across stress, neurodevelopmental, and dopaminergic pathways in relation to pharmacological treatment for NOWS. Sex-specific associations observed in this study further support the possibility that male and female infants differ in their biological responses to prenatal opioid exposure. Significant sex-by-treatment interactions were detected within *DRD4* and *AVP*, indicating that the relationship between methylation and the need for pharmacological treatment for NOWS varied according to infant sex. Within *DRD4*, multiple CpG sites demonstrated higher DNAm in infants who required pharmacological treatment relative to those who did not, regardless of sex, suggesting a general association between increased methylation in this regulatory region and the need for pharmacological treatment. However, the CpG site located −362 bp upstream of the transcription start site demonstrated a sex-specific pattern rather than an overall association with need for treatment. At this specific CpG site, we observed significantly higher DNAm among male infants who required pharmacological treatment with no corresponding difference among females. Together, these findings suggest that different regions within the *DRD4* promoter may be associated with treatment status across all infants, while others may reflect sex-specific responses. Evidence from developmental and behavioral research indicates that the association between *DRD4* genotype and behavioral outcomes often differs between males and females, including male-specific associations with novelty-seeking behaviors and stronger associations with ADHD diagnoses in boys across childhood (Becker et al., 2005; El-Faddagh et al., 2004).

Sex differences in DNAm were also observed within stress regulatory pathways. A significant Sex × Treatment interaction was observed for *AVP*, indicating that the association between treatment status and DNAm differed by infant sex. Female infants who required pharmacological treatment exhibited lower DNAm in *AVP* regulatory regions than those who did not require treatment, although this within-group difference did not reach statistical significance. *AVP* is a gene that

encodes arginine vasopressin, a neuropeptide involved in the regulation of the stress response. Vasopressin signaling plays an important role in hypothalamic stress pathways and may be particularly relevant during prolonged or repeated stress exposure. Sex differences in vasopressin signaling have been reported in stress regulatory brain regions and appear to be influenced by gonadal hormones, suggesting that *AVP* pathways may be differentially regulated in males and females (Sheng et al., 2021; Zuloaga et al., 2020). Because vasopressin expression and signaling are known to be modulated by sex hormones, regulatory regions of *AVP* may be particularly susceptible to sex-specific epigenetic variation. These findings, derived from estimated marginal means, suggest a sex-specific pattern in which treatment-associated differences in DNAm are primarily observed among female infants. Lower DNAm within *AVP* among female infants who required pharmacological treatment for NOWS in the present study may reflect altered regulation of vasopressin signaling involved in stress responsivity. Altered methylation of vasopressin-related genes has also been observed in infants exposed to other substances, including prenatal maternal tobacco use, supporting the sensitivity of this pathway to in utero substance exposures (Nidey et al., 2023).

These findings are consistent with prior work demonstrating sex differences in gene expression across dopaminergic and inflammatory pathways in opioid-exposed neonates (Yen et al., 2019, 2023), supporting the idea that biological sex may influence how stress- and reward-related systems respond to prenatal opioid exposure. Epigenetic regulation of these pathways may represent one mechanism underlying these differences.

The results of this study also have broader implications for understanding the biological mechanisms underlying NOWS heterogeneity. Much of the existing epigenetic literature has focused on the opioid receptor gene *OPRM1*, where increased methylation has been associated with more severe withdrawal and greater need for pharmacological treatment (Wachman et al., 2014, 2018). While this work has provided important insight into opioid receptor signaling, withdrawal symptoms likely arise from dysregulation across several biological systems, including stress physiology, neurobehavioral regulation, and dopaminergic reward signaling. By examining DNAm across multiple pathways within a single cohort, our study expands the scope of candidate gene epigenetic research in NOWS and highlights the potential relevance of dopaminergic and stress-related regulatory mechanisms. Understanding how these pathways contribute to

withdrawal expression may ultimately help identify early biomarkers associated with clinically significant NOWS, which could improve early risk stratification among infants exposed to opioids.

However, an important limitation is that DNAm assays used in this study are currently confined to research settings and are not yet available for routine clinical use for NOWS. In addition, the clinical interpretation of these epigenetic findings remains uncertain, and further validation, standardization, and demonstration of clinical utility would be required before such biomarkers could be implemented to guide care. While epigenetic biomarkers are already used clinically in diseases such as cancer to inform diagnosis and treatment, their application in NOWS remains investigational.

In addition to these translational considerations, several limitations should be considered when interpreting the findings of this study. Although DNAm was measured before the initiation of pharmacological treatment for NOWS, causal inference remains limited. It is unclear whether DNAm differences contribute to the development of NOWS or whether early physiological processes associated with withdrawal influence DNAm patterns. The relatively modest sample size may also have limited the ability to detect small effects, particularly after adjustment for multiple comparisons using FDR correction. Participants were recruited from two clinical sites, which may introduce variability due to site-specific differences in clinical protocols, treatment thresholds, and patient populations. Need for pharmacological treatment was also determined using different clinical assessment approaches across sites, including FNAST, NWI, and ESC, which may further contribute to differences in how treatment need was defined. Although study site was included as a covariate in the statistical analysis, residual confounding related to these differences may remain. Buccal epithelial samples were used to measure DNAm and contain mixed cell populations, meaning that differences in cell type composition may influence DNAm estimates. We were unable to estimate cell type proportions within the scope of the current project. However, this approach is consistent with prior candidate gene studies of prenatal opioid exposure, which relied on bulk peripheral tissues and did not adjust for cell composition (Wachman et al., 2014, 2018). Similarly, we measured buccal epithelial cells rather than neural tissues due to tissue accessibility. While this limits our ability to make causal inferences about dopaminergic or stress pathways in the brain, it is worth noting that both epithelial and neural tissues arise from the same embryonic germ layer (Gilbert, 2000). Buccal cells are therefore commonly used as accessible

peripheral biomarkers in neurodevelopmental epigenetic studies and have been proposed as a useful surrogate tissue for DNAm analyses when target tissues such as the brain are not available (Lowe et al., 2013). In addition, DNAm was measured as a proxy for gene expression, but without downstream gene expression measurements, this limits our ability to infer the functional consequences of observed DNAm differences. Further research examining the functional effects of methylation at these specific CpG sites would be necessary to clarify their biological significance. Finally, our candidate gene approach allowed us to test specific genes and pathways of interest, but our small sample size means that we were only able to evaluate genes' individual associations with NOWS. In future studies, it would be useful to examine interactions between genes and gene networks that contribute to complex neurodevelopmental pathways.

Future research should build on these findings by examining whether DNAm variation at the identified loci is associated with quantitative measures of NOWS severity and related neurobehavioral outcomes. Linking DNAm patterns to clinical measures such as neurobehavioral assessments, maximum Finnegan score, duration of pharmacological treatment, maximum morphine dose, and the use of adjunct medications could help clarify the biological relevance of these epigenetic differences. Larger studies across additional clinical sites will also be important to replicate these findings and better capture the complex relationships between genes involved in stress signaling, neurodevelopment, dopaminergic reward pathways, and opioid receptor signaling. In addition, integrating DNAm data with measures of transcription factor binding and gene expression could further clarify the functional consequences of our observed methylation differences. Ultimately, identifying reproducible epigenetic markers associated with the need for pharmacological treatment of NOWS may help improve understanding of the biological mechanisms underlying withdrawal and support the development of tools that contribute to earlier identification of infants at risk for more severe symptoms.

In conclusion, this study examined whether DNAm in genes involved in dopaminergic signaling, stress regulation, neurodevelopment, and opioid receptor pathways was associated with need for pharmacological treatment among newborn infants with prenatal opioid exposure, and whether these relationships differed by infant sex. Our findings identified associations between DNAm of *DRD4* and *AVP* and need for pharmacological treatment, suggesting that epigenetic regulation of dopaminergic and stress-related pathways, as well as sex-specific biological responses to prenatal

opioid exposure, may contribute to heterogeneity in NOWS. Although these findings require replication and functional validation, they highlight the potential relevance of epigenetic mechanisms in shaping early biological responses to prenatal opioid exposure. Future longitudinal studies integrating DNAm with neurobehavioral outcomes and measures of withdrawal severity will be necessary to determine whether epigenetic variation could serve as a potential predictive biomarker of the need for pharmacological treatment and NOWS severity, and to examine how these early biomarkers may relate to longer-term neurodevelopmental outcomes across early childhood.

Tables

Table 1: Maternal and Infant Characteristics by Infant Pharmacological Treatment Group and Sex

	Overall N = 181	***Treatment N = 76***	***No Treatment N = 105***	***p-value***	***Male N = 89***	***Female N = 92***	
	M (SD) or N (%)	M (SD) or N (%)	M (SD) or N (%)		M (SD) or N (%)	M (SD) or N (%)	***p-value***
Maternal characteristics							
Less than high school degree	45 (17%)	15 (20%)	30 (30%)	0.20	22 (26%)	23 (26%)	1.00
Adequate prenatal care	147 (82%)	63 (83%)	84 (81%)	0.87	74 (83%)	73 (80%)	0.75
No partner	77 (43%)	32 (43%)	45 (43%)	1.00	39 (44%)	38 (42%)	0.85
Hypertension	39 (22%)	19 (25%)	20 (19%)	0.41	17 (19%)	22 (24%)	0.57
Residential Treatment	40 (22%)	15 (20%)	25 (24%)	0.64	19 (21%)	21 (23%)	0.95
Inpatient detox during pregnancy	32 (19%)	12 (17%)	20 (21%)	0.61	14 (17%)	18 (21%)	0.58
Bipolar	47 (26%)	27 (36%)	20 (19%)	0.02*	20 (23%)	27 (30%)	0.40
PTSD	66 (37%)	32 (43%)	34 (33%)	0.25	36 (37%)	30 (33%)	0.31
Hepatitis C	60 (35%)	28 (39%)	32 (31%)	0.35	27 (32%)	33 (37%)	0.60
Infant characteristics							
Sex (Male)	89 (49%)	38 (50%)	51 (49%)	1.00	--	--	--
Gestational age (weeks)	38.29 (1.7)	38.42 (1.8)	38.2 (1.7)	0.41	38.23 (1.7)	38.35 (1.8)	0.65
Birth weight (grams)	3006.41 (551.1)	2984.47 (567.6)	3022.29 (541)	0.65	3077.07 (536.1)	2938.05 (559.6)	0.09
Head circumference (cm)	33.61 (1.9)	33.36 (1.8)	33.8 (1.9)	0.12	33.9 (1.9)	33.34 (1.8)	0.06
Birth length (inches)	48.45 (3.0)	48.28 (3.3)	48.59 (2.9)	0.501	48.86 (3.1)	48.05 (2.9)	0.08
Social Services involvement	85 (47%)	46 (61%)	39 (38%)	0.004*	38 (43%)	47 (52%)	0.29
Breastfeeding	108 (60%)	35 (46%)	73 (70%)	0.003*	50 (64%)	58 (63%)	0.43
Maximum Finnegan Score	10.3 (3.0)	12.34 (2.2)	7.8 (1.8)	<0.001*	10.31 (2.9)	10.29 (3.1)	0.97

Table 2: Summary of Model Results from DRD4 5' Regulatory Region CpG Sites with Significant Main Effects

Distance from TSS	Treatment M (SD)	No Treatment *M* (SD)	*B* Treatment (SE)	Raw *p*-Value	FDR *p*-value
-362	6.36	5.88	0.66 (0.79)	0.40	0.58
-358	0.87	0.88	0.06 (0.13)	0.63	0.68
-351	1.46	1.11	0.47 (0.19)	0.01*	0.10*
-346	4.21	4.33	0.31 (0.48)	0.52	0.61
-342	1.81	1.80	0.22 (0.23)	0.34	0.56
-338	0.86	0.87	0.31 (0.15)	0.04*	0.10*
-333	0.66	0.98	-0.27 (0.12)	0.03*	0.10*
-321	2.06	2.11	0.01 (0.30)	0.97	0.97
-315	1.68	1.31	0.23 (0.24)	0.34	0.56
-312	0.86	0.63	0.07 (0.11)	0.51	0.61
-305	2.02	1.87	0.42 (0.28)	0.13	0.27
-303	1.59	1.19	0.43 (0.19)	0.02*	0.10*
-301	3.53	2.95	0.87 (0.39)	0.03*	0.10*

Note: Treatment and no treatment M (SD) values represent unadjusted means. β coefficients are adjusted estimates from robust linear regression models controlling for infant sex and study site.
*Indicates $p < 0.05$ or FDR adjusted ≤ 0.1

Figures

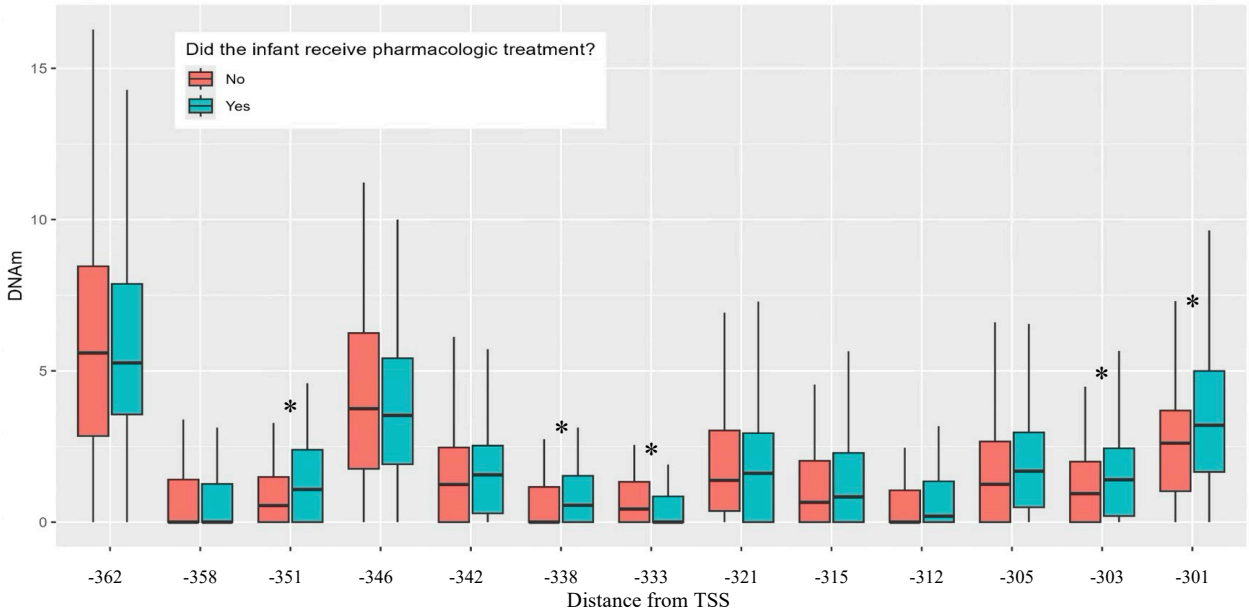

Figure 1: DNA methylation levels at DRD4 CpG sites by pharmacological treatment status

Note: *indicates FDR adjusted ≤ .10

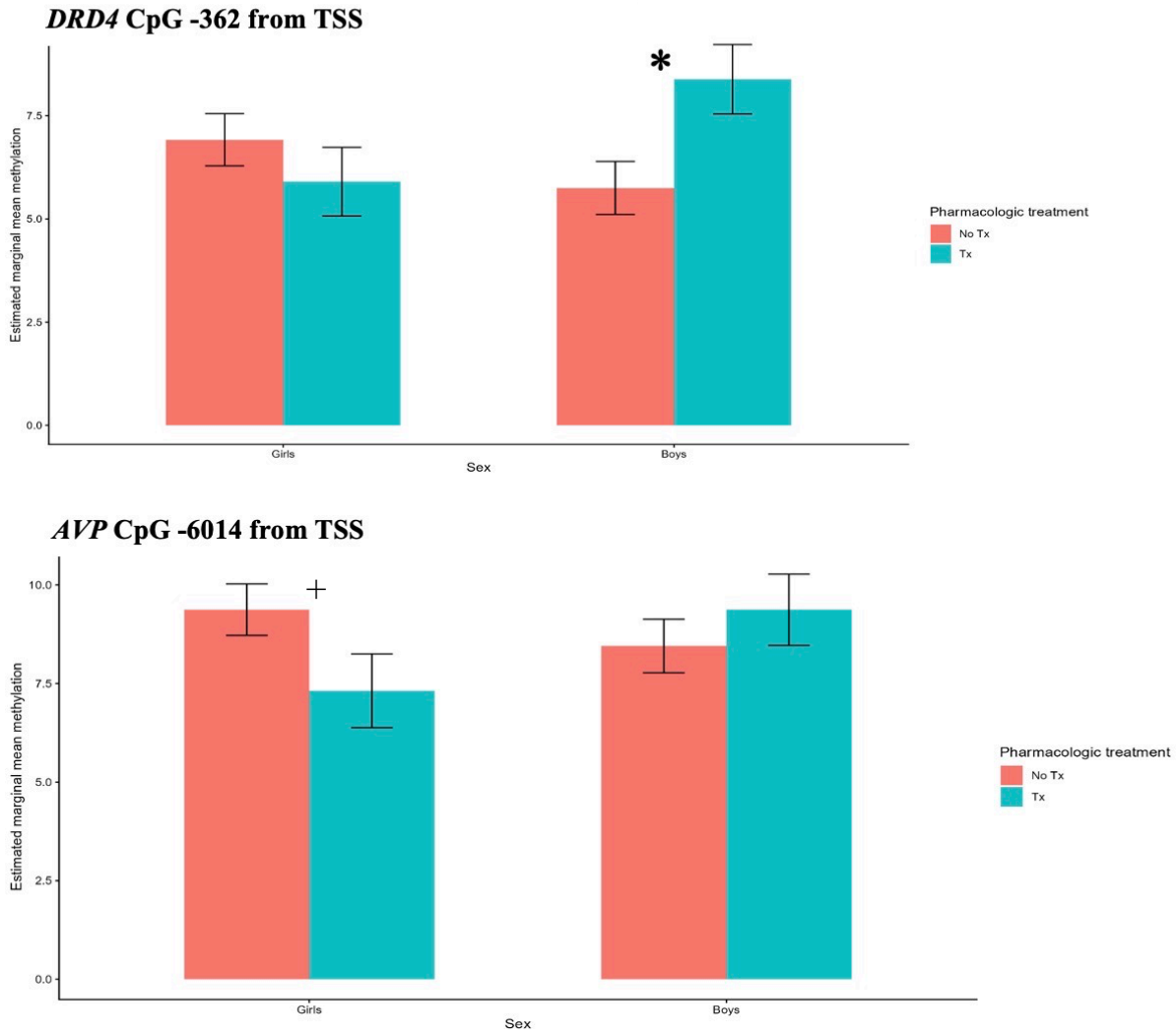

Figure 2: Estimated marginal means illustrating Sex × Treatment interactions at Significant CpG sites

Note: *$p < 0.05$; + $p = 0.06$

Supplementary

Table 1: Maternal and Infant Characteristics by Inclusion Status

	Overall ***N = 235***	***Included*** ***N = 181***	***Excluded*** ***N = 54***	
	M (SD) or N (%)	M (SD) or N (%)	M (SD) or N (%)	***p*-value**
Maternal Characteristics				
Less than high school degree	57 (0.25%)	45 (25.9%)	12 (22.6%)	0.77
Adequate prenatal care	189 (82%)	146 (81.1%)	43 (84.3%)	0.87
No partner	107 (46%)	77 (43%)	30 (56.6%)	0.11
Hypertension	49 (21%)	39 (21.7%)	10 (18.9%)	0.80
Residential Treatment	47 (20%)	40 (22.1%)	7 (13%)	0.20
Inpatient detox during pregnancy	41 (19%)	34 (20%)	7 (15.2%)	0.60
Bipolar	52 (22%)	47 (26.4%)	5 (9.4%)	0.02*
PTSD	85 (37%)	66 (37.1%)	19 (35.8%)	1.00
Hepatitis C	73 (32%)	60 (34.7%)	13 (24.5%)	0.22
Infant Characteristics				
Sex (Male)	120 (51%)	89 (49.2%)	31 (57.4%)	0.36
Gestational age (weeks)	38.25 (1.71)	38.29 (1.74)	38.12 (1.65)	0.52
Birth weight (grams)	3015.9 (542.6)	3006.41 (551.08)	3047.85 (517.03)	0.61
Head circumference (cm)	33.8 (2.19)	33.61 (1.88)	34.27 (2.96)	0.14
Birth length (inches)	48.4 (3.24)	48.45 (2.99)	48.05 (4.03)	0.52
Social Services involvement	114 (49%)	85 (47.2%)	29 (54.7%)	0.42
Breastfeeding	138 (59%)	108 (59.7%)	30 (55.6%)	0.70
Maximum Finnegan Score	10.1 (3.01)	10.3 (3.01)	9.26 (2.94)	0.08

Note: *$p < 0.05$

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