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ORIGINAL ARTICLE

Cannabis use by women during pregnancy does not influence infant DNA methylation of the dopamine receptor *DRD4*

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ABSTRACT

Background: Maternal cannabis use in pregnancy is linked with long-term adverse behavioral outcomes in offspring. Epigenetic processes established *in utero* that affect dopaminergic (reward) signaling may mediate risks. Associations between cannabis use and offspring DNA methylation have not been investigated; however, maternal tobacco smoking in pregnancy is associated with distinct patterns of DNA methylation at birth and beyond. *Objectives*: To determine whether maternal cannabis use is associated with methylation of the dopamine receptor gene *DRD4* promoter in infants. *Methods*: Mothers in the Triple B study provided detailed information on drug use in each trimester of pregnancy. Buccal swabs were collected from neonates at 8 weeks (n = 804, 51.7% male, and 48.3% female). DRD4 promoter DNA methylation was measured using SEQUENOM MassARRAY. *Results*: Fifty-seven of the women in the study reported drug use during pregnancy, of whom 44 used cannabis. Of 19 cytosine-phosphate-guanine dinucleotides (CpG) units tested in DRD4, gestational cannabis use was associated with offspring methylation at 1 CpG unit in multivariate models (β + 1.48, CI: 0.02 to 2.93, and p = 0.047). At another site there was weak evidence that both cannabis and other drug use were independently associated with increased methylation, while the association with tobacco was in the reverse direction (cannabis use β + 0.67, CI: −0.12 to 1.46, and p = 0.09; other drug use β + 1.11, CI: 0.17 to 2.05, and p = 0.02; tobacco use β −0.41, CI: −0.85 to 0.03, and p = 0.07). None of the associations would remain significant after correction for multiple testing. *Conclusion*: There is no strong evidence that maternal cannabis use in pregnancy is associated with offspring DRD4 methylation.

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Introduction

Drug or "substance" use during pregnancy is relatively common, reported by up to 5% of women in the general population, with cannabis the most frequently used (1). Given the changing prevalence of use related to legislative changes in some countries (i.e., increasing use with decriminalization), studies concerning maternal cannabis use are an important consideration for public health (2,3).

Although research into the effects of prenatal exposure to maternal cannabis use on offspring is still in its infancy, adverse effects have been reported. A recent meta-analysis involving 24 studies found that cannabis-exposed infants had a higher risk of anemia, decreased birth weight, and a greater chance of being placed in intensive care (4). Subsequent studies have reported similar findings (5) as well as longer-term detrimental effects. Mothers who used cannabis daily in the first trimester had offspring with deficits in verbal reasoning and short-term memory in childhood (6) and any *in utero* exposure has been associated with attention problems and increased aggression in 18-month-old girls (7). Heavy cannabis use (one or more joints a day) during the first trimester of pregnancy has also been associated with lower reading, spelling, maths, and comprehension skills in 10-year-old primary school

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students (8), which may be due in part to an increased risk of mood disorders such as anxiety and depression.

The detrimental effects of cannabis could result from the transmission of Δ^9-tetrahydrocannabinol (THC), the psychoactive constituent within cannabis, to the fetus (9). THC can readily cross the placenta, it has been shown to increase the diameter of umbilical veins and arteries, and THC stimulation of endocannabinoid signaling may induce apoptosis of developing placental cytotrophoblasts (10). Thus biologically available THC can have a negative impact on the fetus *in utero*. Given its key role in prenatal development and association with several postnatal phenotypes in humans, epigenetic mechanisms may help mediate these short-term and long-term detrimental effects (11). Broadly, epigenetics refers to environmentally sensitive alterations in gene expression that do not change the underlying DNA sequence (12). DNA methylation, particularly methylation of cytosine-phosphate-guanine dinucleotides (CpG), is the most commonly studied epigenetic mechanism.

Most epigenetic studies of cannabis have focused on the effects of cannabis use in adults, primarily investigating the THC cannabinoid receptor type 1 (*CNR1*) which encodes the receptor CB_1R. Elevated methylation of the *CNR1* promoter and downregulation of CB_1R mRNA, as measured in peripheral blood, is associated with cannabis use in schizophrenia patients (13). Methylation levels were also related to the severity and frequency of cannabis cravings. CB_1R is known to be co-expressed with dopamine receptors (14) and cannabis, like all recreational drugs, causes an increase in dopamine levels in the terminal regions of the mesolimbic dopamine system. Dopamine signaling is linked to memory, cognition, and is one of the major reward pathways in the brain implicated in drug addiction (15). Little is known about the effects of maternal cannabis use on methylation of genes involved in dopamine signaling in offspring.

The primary aim of this study was to determine the association between maternal gestational cannabis use and offspring DNA methylation. Of the set of genes controlling neuro-signaling within dopaminergic pathways, this study focused on offspring *DRD4* methylation patterns given that this gene has not only been associated with substance use (16) and addiction (17,18), but importantly has also been linked to infant health outcomes, including birth weight, behavior, and neurodevelopment (19,20). Differential methylation of this gene could thus help mediate the association between maternal substance use and poor infant health outcomes, although no studies to date have yet measured *DRD4* methylation in this regard.

The secondary aim was to investigate how maternal tobacco smoking could influence the association between cannabis and infant methylation. Most studies to date do not differentiate cannabis from tobacco use, despite the fact that cannabis is often smoked together with tobacco, and tobacco has similar effects on infant health. This is problematic because maternal smoking during pregnancy has been consistently shown to influence infant DNA methylation in peripheral tissue and to mediate the association between prenatal exposure (21,22) and infant birth weight (23). Furthermore, a very recent study found that cannabis alone had no independent effect on perinatal outcomes, but augmented the risk associated with tobacco smoking (24). Studies examining effects associated with tobacco and cannabis in isolation and in combination are needed to elucidate unique effects.

Methods

Triple B pregnancy cohort

This longitudinal pregnancy cohort of 1,634 families is focused on perinatal maternal lifestyle factors and infant health outcomes. Families were recruited through general public and specialist drug and alcohol antenatal services at major hospitals in New South Wales and Western Australia (25). Mother–infant dyads with major medical complications were excluded. Written informed consent was provided by all participants, and all relevant human research ethics committees approved the study. In-depth health, lifestyle, and sociodemographic data were collected across pregnancy and postpartum. This included detailed questionnaires about drug use in each trimester of pregnancy and the first 8 weeks postpartum.

Methylation analysis

The *DRD4* methylation assay covered a 396 base pair region of the gene promoter spanning chr11:635,510–636,905 (UCSC hg38), previously shown to be differentially methylated in association with attention deficit hyperactivity disorder (ADHD) (26). Infant buccal swabs were collected at 8 weeks of age. Extracted DNA underwent bisulfite conversion (EZ-96 DNA Methylation-Lightning™ MagPrep kit (Irvine, CA)), followed by PCR amplification in triplicate with primers 5′-GGACCCCCTGCCCAGGGTCAGAGG-3′ and 5′-TGCCAGATACCAGGTGGACTAGGGTG-3′, and Sequenom MassARRAY analysis (27). Average *DRD4* methylation was calculated after excluding any triplicate samples deviating by ≥10% from the median.

Outlying data (>10% outside 1.5xIQR) were excluded, as were CpG units (n = 4) and participants with <85% data retained after quality control (n = 92). Health, lifestyle, and sociodemographic data did not differ significantly between excluded participants and those included in this analysis.

Statistical analysis

To investigate the association between prenatal maternal substance use (yes/no) with infant *DRD4* promoter methylation, t-tests were used for univariate analysis, followed by multivariate linear regression models adjusted for potential confounding factors, and Sequenom batch effects.

Results

Table 1 shows the maternal and infant characteristics of the population according to cannabis use during pregnancy. Cannabis use was the most commonly used drug (n = 44, 77.2% of drug users) and was most common among mothers in trimester 1 (Table 2), likely prior to knowledge of pregnancy. Of those who reported cannabis use, eight used cannabis daily throughout trimester 1, with the dose ranging between (the equivalent of) 0.5 and 7 joints. A total of 29 women used other types of drugs, with the type and quantity varying quite considerably. Of these women, 16 also used cannabis.

We first investigated the association between any type of substance use during pregnancy and infant *DRD4* promoter methylation. Of 19 CpG units tested, the only evidence of an association was a very small increase in methylation at CpG.32 (Δ + 0.78, 95% CI: 0.17–1.38%, and p = 0.012) (Figure 1, Table S1). Cannabis use was similarly associated with differential methylation at the same site (Δ + 0.79, 95% CI: 0.11–1.46%, and p = 0.023) (Figure 2, Table S2). These associations remained even after adjustment for covariates, including drug use at 8 weeks postpartum, given the potential for effects resulting from exposure during breastfeeding or second-hand exposure. Smoking tobacco during pregnancy was itself associated with *DRD4* methylation at this same site, but in the reverse direction (cannabis: β + 0.77, 95% CI: 0.06 to 1.48, and p = 0.032; tobacco: β −0.41, 95% CI: −0.85 to 0.03, and p = 0.072). However, when adjusting for other substance use as a covariate, which was also associated with increased CpG.32 methylation, the association with cannabis becomes nonsignificant (cannabis: β + 0.67, 95% CI: −0.12 to 1.46, and p = 0.098; other substance use: β + 1.11, 95% CI: 0.17 to 2.05, and p = 0.020). Cannabis use was also associated with increased methylation at CpG.21.22.23 when we adjusted for tobacco use in the multivariate model (β + 1.48, 95% CI: 0.02 to 2.93, and p = 0.047, unadjusted Δ + 1.03, 95% CI: −0.29% to 2.36%, and p = 0.13), and regardless of other drug use. Out of interest, adding alcohol use during pregnancy to these models did not alter the associations seen.

Table 1. Maternal and infant population characteristics according to maternal cannabis use during pregnancy (N = 804*).

Characteristic	Cannabis use during pregnancy	
	No (n = 760)	Yes (n = 44)
Maternal	Mean ± SD	
Age at child's birth (years)	32.6 ± 4.8	30.2 ± 6.5
Country of birth:	*N* (%)	
Australia	418 (55.0)	36 (81.8)
Other	342 (45.0)	8 (18.2)
Currently living with partner	720 (94.7)	29 (65.9)
Education level:		
Year 12 or under	124 (16.3)	25 (56.8)
Completed TAFE[a]/tech school[b]	104 (13.7)	9 (20.5)
Completed university/college	532 (70.0)	10 (22.7)
Employment:		
Full time/self-employed	362 (47.6)	11 (25.0)
Part time/casual	145 (19.1)	5 (11.4)
Home/student/unemployed	253 (33.3)	28 (63.6)
Substance use during pregnancy:		
Tobacco use	100 (13.2)	35 (79.6)
Alcohol consumption	514 (67.6)	35 (79.6)
Infant		
Sex:		
Male	389 (51.3)	26 (59.1)
Female	370 (48.7)	18 (40.9)
	Mean ± SD	
Gestational age (weeks)	39.4 ± 1.6	39.0 ± 2.0
Birth weight (kg)	3.5 ± 0.5	3.3 ± 0.7

[a]TAFE: Technical and further education institutions.
[b]Technical school: Similar to TAFE but more trade oriented.
*Except for infant sex and gestational age (n = 803), and birth weight (n = 801).

Table 2. Maternal substance use across pregnancy and at 8 weeks postpartum.

	N (%)				
Drug	Trimester 1	Trimester 2	Trimester 3	Any time during pregnancy	8 weeks postpartum
Cannabis	44 (5.5)	19 (2.4)	11 (1.4)	44 (5.6)	15 (1.9)
Heroin	8 (1.0)	3 (0.4)	2 (0.3)	8 (1.0)	0 (0)
Non-prescribed opioids	1 (0.1)	1 (0.1)	0 (0)	1 (0.1)	1 (0.1)
Amphetamines	11 (1.4)	1 (0.1)	0 (0)	13 (1.5)	1 (0.1)
Cocaine	10 (1.2)	0 (0)	0 (0)	11 (1.2)	1 (0.1)
Hallucinogens	3 (0.4)	1 (0.1)	0 (0)	3 (0.4)	0 (0)
Club drugs	5 (0.6)	0 (0)	0 (0)	5 (0.6)	0 (0)
Total	56 (7.0)	20 (2.5)	9 (1.1)	57 (7.1)	16 (1.9)

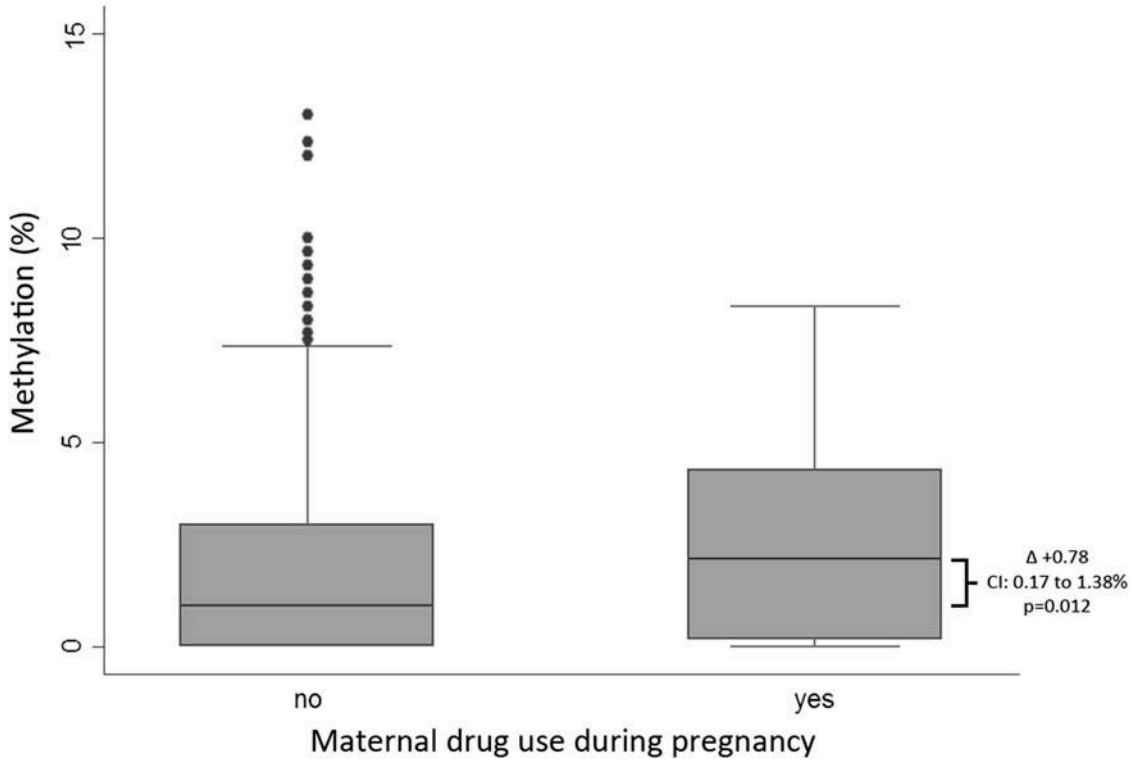

Figure 1. Methylation of infant *DRD4* CpG.32 according to maternal substance use during pregnancy.

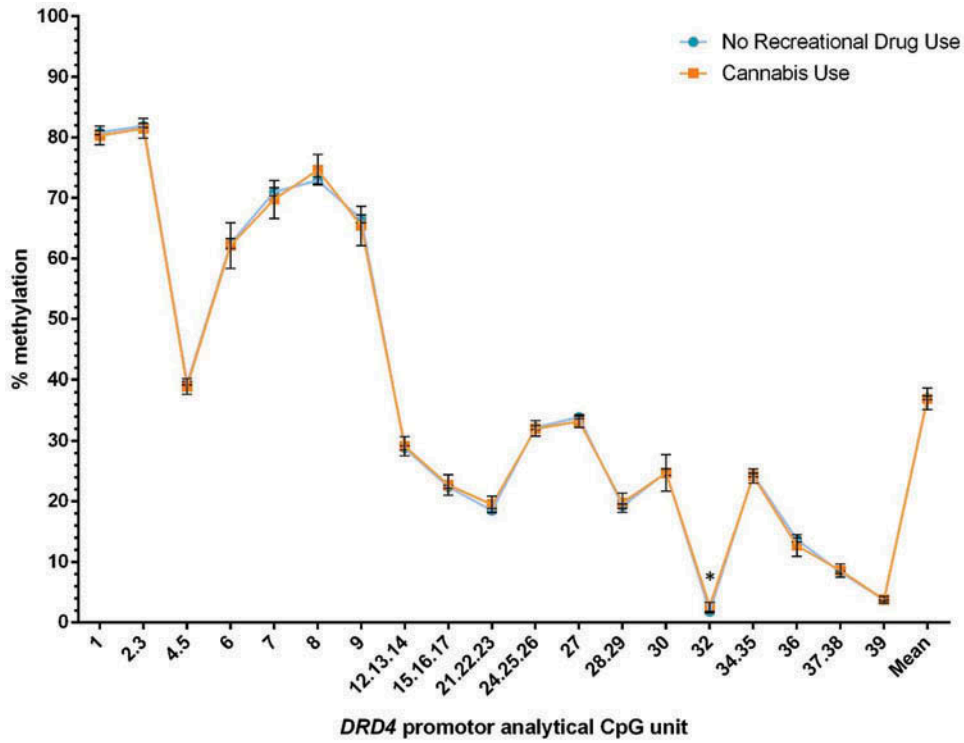

Figure 2. Mean infant *DRD4* promoter methylation according to maternal cannabis use during pregnancy. Bars indicate 95% CI.

Table 3. Infant *DRD4* CpG.32 methylation when comparing substance use groups to no substance use during pregnancy.

Substance group	Difference (%)	95% CI	*p* Value
Smoking only	−0.36	−0.99 to 2.65	0.45
Cannabis only	1.71	−0.09 to 3.52	0.069
Both smoking and cannabis	0.59	−0.38 to 1.56	0.40

Given that cannabis is frequently taken together with tobacco, we further compared *DRD4* promoter methylation levels across four groups: no drug or tobacco use (n = 658), tobacco alone (n = 96), cannabis alone (n = 9), and both cannabis and tobacco (n = 35). Only cannabis use appeared to increase methylation compared to no substance use (Table 3). In addition, exclusive cannabis use was associated with 2.08% higher methylation at CpG.32 compared to tobacco use alone (95% CI: 0.19–3.96% and p = 0.024). Exclusive cannabis use compared to combined cannabis and tobacco showed no significant difference in DNA methylation (p = 0.48).

Discussion

Here we report a nominally significant association between cannabis use during pregnancy and increased *DRD4* promoter methylation at one CpG site (CpG11:636796, hg38) in infants, which remained after adjustment for concurrent tobacco use. We also found some, although nonsignificant, evidence that both other drug use and cannabis use appeared to increase methylation at another site, while tobacco smoking had a reverse effect (decreased methylation). This is despite reports that cannabis and tobacco have similar (28) and even cumulative detrimental effects on infant health (24). However, the overall effect sizes observed are very small and the strength of association would not remain if corrected for multiple testing (using a Bonferroni corrected significance level of 0.0026 given the 19 CpG units examined).

Dopamine signaling is involved in many cognitive processes, including memory and reward (15). Cannabis dependence is linked to a reduction in striatal dopamine release which is also correlated with poor working memory and learning performance (29). Prenatal cannabis exposure has been associated with decreased dopamine receptor D2 expression in human fetal brain specimens (30). Dopamine receptor deficiency has been linked to increased choice and immediacy to use drugs, and is observed in people with substance use disorders (31). *DRD4*, a D2-like dopamine receptor, was chosen as a strong candidate gene in this study due to its key role in dopamine signaling, and its links to drug use in genetic association studies. A *DRD4* variable number tandem repeat has been associated with increased problematic cannabis use (32), and a heightened risk of externalizing behavior problems that contribute to substance use disorders and addiction (33). *DRD4* genetic variants can also moderate the effect of prenatal stress on children's antisocial behavior (34). Furthermore, increased *DRD4* methylation in saliva has been associated with increased severity of ADHD symptoms in children (35).

Our study is strengthened by the in-depth data concerning substance use and tobacco smoking across pregnancy and the large sample size. The percentage of women using drugs during pregnancy (7.03%) was higher than that reported in the general Australian population (2.2%) (36). Our study had over 80% power (α = 0.05, two-sided) to detect a minimum effect size of 0.385 standard deviation (SD) difference in the mean methylation between groups. For CpG.32 for example, with a group SD of 2.2, our study could detect a methylation difference of 0.86% between groups defined by drug use. The power of the analysis was nevertheless limited, particularly in regard to the timing or dose of exposure during pregnancy, which could not be investigated, or specific drugs other than cannabis, which are likely to have varying effects on methylation and infant health outcomes (37). Tissue specificity of DNA methylation marks may contribute to the largely negative findings of our study. Dopamine signaling is prominent in the brain, but due to the limitations in accessing such tissue, we chose to investigate buccal cell DNA methylation, given that (1) buccal epithelial cells have the same developmental origins to neuronal cells (i.e., derive from ectoderm) and (2) previous studies have provided some support for the use of buccal cells as a proxy for neurodevelopmental phenotypes (38). The power of the study may have been limited due to underreporting of maternal substance use, which could help explain the predominantly negative findings (if a proportion of women classified as nonusers had in fact used drugs during pregnancy). However, to address this limitation in the Triple B study, a random selection of 85 participants provided urine samples for analysis in the third trimester of pregnancy. There was 97% agreement between self-report substance use and urine analysis, indicating the overall high accuracy of the self-report data. Other limitations are that we could not properly

investigate the independent effects of tobacco from cannabis, as only nine cannabis users in the study did not smoke tobacco.

Conclusion and future research

We found no convincing evidence of an association between prenatal substance use overall or cannabis use and differential infant *DRD4* methylation. However, given the reported effects of maternal drug use on infant health outcomes, as well as consistent evidence that smoking tobacco during pregnancy influences infant peripheral DNA methylation, it is possible that genes other than *DRD4* may be involved, such as *CNR1*. Further studies could investigate other components of dopaminergic signaling, or use a more unbiased discovery approach such as an epigenome-wide association study. Both approaches would benefit from a substantially larger sample of maternal drug users. Such approaches are essential to provide a better understanding of the biological mechanisms underlying the association between prenatal drug exposure and infant health outcomes.

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Declaration of interest

The authors declare no conflicts of interest.

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