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RESEARCH ARTICLE

Perinatal maternal alcohol consumption and methylation of the dopamine receptor *DRD4* in the offspring: the Triple B study

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Abstract

Maternal alcohol use during the perinatal period is a major public health issue, the higher ends of which are associated with foetal alcohol spectrum disorder and a range of adverse health outcomes in the progeny. The underlying molecular mechanisms remain largely unknown but may include the epigenetic disruption of gene activity during development. Alcohol directly activates the neurotransmitter dopamine, which plays an essential role in neurodevelopment. To investigate whether antenatal and early postnatal alcohol consumption were associated with differential dopamine receptor *DRD4* promoter methylation in infants ($n = 844$). Data were drawn from the large population based Triple B pregnancy cohort study, with detailed information on maternal alcohol consumption in each trimester of pregnancy and early postpartum. DNA was extracted from infant buccal swabs collected at 8-weeks. *DRD4* promoter DNA methylation was analysed by Sequenom MassARRAY.

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No strong evidence was found for an association between alcohol consumption during pregnancy and infant *DRD4* methylation at 8-weeks postpartum. However, maternal alcohol consumption assessed contemporaneously at 8-weeks postpartum was associated with increased methylation at 13 of 19 CpG units examined (largest $\Delta + 3.20\%$, 95%Confidence Interval:1.66,4.75%, $P = 0.0001$ at CpG.6). This association was strongest in women who breastfeed, suggesting the possibility of a direct effect of alcohol exposure via breast milk. The findings of this study could influence public health guidelines around alcohol consumption for breastfeeding mothers; however, further research is required to confirm these novel findings.

Key words: alcohol; pregnancy; perinatal; postpartum; dopamine receptor (DRD4); foetal programming; DNA methylation; epigenetics; breastfeeding

Introduction

Australian prevalence data suggest that alcohol use during pregnancy is common [1]. A recent National Drug Strategy Household survey indicated that over 50% of women drank alcohol prior to pregnancy awareness, and one in four continued to drink throughout pregnancy [2]. High maternal alcohol use during pregnancy is associated with a range of detrimental short- and long-term effects on the developing foetus, such as increased risk of birth defects, delayed development and poor child behaviour [3, 4]. This may occur through foetal programming, where the foetus adapts to its intrauterine environment to maximize its growth and development *in utero*, impacting later health [5]. Alcohol is a known teratogen, and the foetus is directly exposed to maternal alcohol consumed during pregnancy via diffusion across the feto-placental barrier [6]. This can directly influence foetal development [7]. Accumulating evidence suggests that epigenetic mechanisms, including DNA methylation, may play a mediating role in foetal programming. Epigenetics refers to mitotically heritable alterations to cellular phenotype or gene expression that do not change the underlying DNA sequence [8]. Numerous maternal exposures in pregnancy, such as stress, smoking and folate intake, have now been associated with differential DNA methylation in more than one study [9–11].

Few studies have investigated the influence of *in utero* alcohol exposure on infant DNA methylation. One epigenome-wide association study (EWAS) using buccal swab DNA of children with foetal alcohol spectrum disorder ($n = 6$), identified a number of genes that were differentially methylated between cases and controls, particularly genes involved in neurodevelopment [12]. In terms of more moderate levels of alcohol consumption, such as a few standard drinks a week which is commonly observed in the general population, only a few candidate gene studies have been undertaken. Prenatal alcohol consumption has been associated with an increase in methylation (3.3%) of the long interspersed nuclear Element-1 (*LINE1*) in placenta ($n = 380$), compared with non-drinkers [13]; however, the sample size of women reporting prenatal drinking ($n = 3$) was severely limited. Another study showed that pleiomorphic adenoma gene-like 1 gene (*PLAGL1*) in cord blood was differentially methylated in association with prenatal alcohol use in Trimester 3 ($n = 254$) [14], although effect sizes were not reported. In a study of 164 cord blood samples, maternal binge drinking during pregnancy was associated with an ~20% increase in methylation at dopamine associated transporter (*DAT1*) promoter compared with non-binge drinkers (all other alcohol groups combined including heavy drinkers) [15]. No data on the timing of alcohol consumption were available though, and there was no difference in *DAT1* methylation between non-drinkers and drinkers overall. Genes involved in dopaminergic signalling, however, do represent good candidates for disruption in response to alcohol exposure.

Motivation to consume alcohol is, in part, thought to be mediated by reward pathways within the meso-cortico-limbic system of the mid brain. Dopamine is the primary neurotransmitter within this system and alcohol directly activates dopaminergic signalling [16, 17]. Dopaminergic signalling also plays an important role in mood regulation and neurodevelopment [18 19]. Disruptions in dopaminergic signalling can result in a broad range of neuropsychiatric and development conditions, including depression and schizophrenia [20]. The dopamine receptor D4 (*DRD4*) was chosen as a strong candidate gene for investigation in this study given that it is a key component of dopamine signalling, responsible for inhibiting adenylate cyclase during signal transduction [21]. Furthermore, higher peripheral *DRD4* methylation levels have been observed in in adults with alcohol dependence [22] but no study has yet investigated whether perinatal alcohol exposure can influence *DRD4* methylation in the offspring.

The aim of this study was to examine the association between maternal alcohol intake during pregnancy and early postpartum, and infant buccal *DRD4* promoter methylation shortly after birth, taking into account the timing of alcohol exposure and a range of possible confounding perinatal factors.

Methods

Triple B Cohort

Triple B is a longitudinal pregnancy cohort study that recruited 1634 families though public antenatal services associated with major hospitals in New South Wales and Western Australia, including specialist drug and alcohol antenatal services. Mothers and or pregnancies with major medical complications were excluded. Ethics approval for the study was granted by the relevant Human Research Ethics Committees, and all participants provided written informed consent. Participants responded to questionnaires to gather data on socio-demographic, health and lifestyle factors across pregnancy and postpartum. Women self-reported alcohol consumption in each trimester of pregnancy and during the first 8-weeks postpartum, by completing detailed questionnaires. To address a potential bias in the self-reporting of substance use, a random selection of participants ($n = 85$) underwent a urine analysis during the third trimester. Between self-reporting and urine analysis, the data were 97% in agreement, which indicated the strong reliability of the reporting.

DRD4 Promoter Region

The 396 bp assay designed using epidesigner.com, covered a region of the promoter CpG island (chr11:635 510–636 905,

University of California, Santa Cruz (UCSC) genome build hg38), previously shown to be differentially methylated [23, 24] (forward primer 5′-GGACCCCCTGCCCAGGGTCAGAGG-3′; reverse 5′-TGCCAGATACCAGGTGGACTAGGGTG-3′). Data were obtainable for 19 analytical CpG units encompassing 32 CpG sites. DNA was extracted from 8-week infant buccal samples and 400 ng of genomic DNA was used for bisulphite conversion [EZ-96 DNA Methylation-Lightning MagPrep kit (Irvine, USA)]. All samples were amplified and assayed in triplicate. DNA methylation data were obtained using the Sequenom MassARRAY EpiTYPER. Technical replicates passed quality control if they were within 10% of the median sample, and the average methylation was calculated. CpG units or participants where <85% of methylation data were obtained after quality control, as well as outliers (>10% outside 1.5 times the Interquartile Range) were excluded (Supplementary Table S1).

Statistical Analysis

From 903 extracted infant buccal samples, 844 passed quality control (technical triplicates within 10% of sample median at each CpG, and >85% data retained for all CpGs per sample). For each individual CpG unit, sample number ranged from 743 to 844 (Supplementary Table S1), with 552 samples having complete data for all 19 CpG units. These participants were representative of the whole sample, as they did not differ significantly on the socio-demographic, health and lifestyle characteristics shown in Table 1.

Univariate analysis was used to investigate the association between alcohol exposure during pregnancy and mean *DRD4* methylation. The association between maternal alcohol consumption at 8-weeks postpartum and mean infant *DRD4* methylation at this same time-point was also examined, as were associations between alcohol exposure and methylation levels at individual CpG units. When significant associations were found, multivariate linear regression models were generated including potential confounding factors, associated with both alcohol consumption and *DRD4* methylation. Batch effects from different Sequenom Chips were controlled for.

Table 1. Maternal and infant population characteristics

Population characteristics		
Maternal characteristics	*n*	Mean ± SD
Maternal age at child's birth (years)	821	32.6 ± 5.0
Country of birth	844	*n*(%)
Australia		464 (55.0)
Other		380 (45.0)
Currently living with partner	821	762 (92.8)
Education level	821	
Year 12 or under		153 (18.6)
Completed Tafe/tech school		116 (14.1)
Completed university/college		552 (67.3)
Employment	821	
Full-time/self-employed		377 (45.9)
Part time/casual		154 (18.8)
Home/student/unemployed		290 (35.3)
Maternal alcohol consumption		
At any time during pregnancy	816	561 (68.8)
Trimester 1	816	507 (62.1)
Trimester 2	821	254 (30.9)
Trimester 3	811	254 (31.3)
8-weeks postpartum	814	502 (61.7)
Maternal tobacco use		
Any time during pregnancy	812	139 (17.1)
8-weeks postpartum	814	77 (9.5)
Infant characteristics		
Sex: male	816	422 (51.7)
Female		394 (48.3)
		Mean ± SD
Gestation age at birth (weeks)	816	39.3 ± 1.7
Birthweight (kg)	812	3.5 ± 0.5

Results

Study Population

The maternal and infant characteristics of the study population are shown in Table 1. The majority of women consumed alcohol during pregnancy, predominantly in Trimester 1 prior to pregnancy awareness. There was a similar frequency of drinkers at 8-weeks postpartum (61.7%).

Maternal Alcohol Consumption during Pregnancy and Infant DRD4 Methylation

We first examined whether maternal alcohol use during pregnancy and early postpartum was associated with differential *DRD4* methylation in infants at 8-weeks (Table 2). Mothers who reported drinking alcohol during Trimester 3 (64.6% of whom also drank in both Trimesters 1 and 2) had infants with significantly higher *DRD4* methylation levels, both mean levels across the region (Table 2) and at individual 5 of 19 CpG units (Supplementary Table S2). Postpartum alcohol was also significantly associated with increased mean *DRD4* methylation and at 13 of 19 units, (Table 3 and Supplementary Fig. S1). In the latter case, 5 of these associations would remain significant even after Bonferroni correction taking into account the 19 CpG units examined ($P < 0.0026$). The largest effect size observed was at CpG.6 (chr11:636 593) ($\Delta + 1.87$, 95%CI:0.21,3.52%, $P = 0.027$ Trimester 3; $\Delta + 3.20$, 95%CI:1.66,4.75%, $P = 0.0001$ 8-week postpartum, Fig. 1).

Given that significant associations were identified with both maternal alcohol consumption during Trimester 3 and early postpartum, we sought to disentangle these effects developmentally. It was not possible to isolate the effects of drinking at Trimester 3, because with the exception of 1 participant, all mothers who drank in Trimester 3 also drank at 8-weeks postpartum. Furthermore, only 6% of women drinking in Trimester 3, abstained from drinking at other stages of pregnancy. However, we could investigate the subpopulation of women who abstained from drinking alcohol during Trimester 3 but drank early postpartum ($n = 161$). Indeed, when excluding Trimester 3 drinkers from the analysis, the strength of association between early postpartum alcohol consumption and *DRD4* methylation increased in effect size Supplementary Table S3, CpG.6: 4.87%, $P = 0.0007$.

Multivariate Analysis

As shown in Table 4, women who reported alcohol consumption postpartum were older, more likely to be Australian born, more highly educated and employed full-time. Infants exposed to alcohol 8-weeks postpartum were more likely to have had a longer gestation age and a heavier birthweight. Maternal age ($P = 0.11$) and education level ($P = 0.10$) also showed moderate associations with *DRD4* methylation, as did tobacco use during pregnancy ($P = 0.11$). *DRD4* promotor methylation also higher in male infants compared with females ($P = 0.005$). There was no

Table 2. association between maternal alcohol consumption and infant mean *DRD4* promoter methylation

Alcohol consumption			*DRD4* methylation		
		n	Mean methylation (95%CI)	Δ	*P*
At any stage during pregnancy	No	161	36.98 (36.74,37.56)	+0.13	0.704
	Yes	375	37.11 (36.75,37.48)		
During Trimester 1[a]	No	195	37.09 (36.55,37.62)	+0.05	0.88
	Yes	339	37.03 (36.66,37.41)		
During Trimester 2[a]	No	367	36.96 (36.59,37.33)	+0.37	0.28
	Yes	172	37.32 (36.77,37.88)		
During Trimester 3[a]	No	373	36.83 (36.46,37.20)	+0.89	0.0090
	Yes	161	37.72 (37.17,38.26)		
During the first 8-weeks postpartum[a]	No	202	36.51 (36.02,37.00)	+0.91	0.0049
	Yes	334	37.41 (37.02,37.80)		

[a]Women's reporting of consumption at that period but not exclusively (i.e. 62% of women consuming alcohol in Trimester 3, also reported consumption in Trimesters 1 and 2).

significant association between breastfeeding and alcohol consumption early postpartum ($P = 0.66$). We therefore adjusted for maternal age, education level, plate number (to account for possible batch effects) and in multivariate models, while also ensuring that the inclusion of other covariates such as offspring sex, did not modify the findings. Smoking was included in the adjusted models given the strong published evidence that it can influence DNA methylation in the offspring [25]. However, inclusion of these covariates had no influence on the findings (i.e. 8-week alcohol consumption, (mean β:+0.80, SE:0.32, $P = 0.014$; CpG.6 $\beta + 2.88$, SE 0.79, $P < 0.001$)).

The Potential Role of Breastfeeding in Alcohol Transmission

To further investigate our principal finding, that of an association between alcohol exposure during the first 8-weeks postpartum and an increase in infant *DRD4* methylation assessed contemporaneously, we considered the possible role of breastfeeding. As all participant data were not available at the time of this study, we focused on the subpopulation of women who reported breastfeeding for the first 8-weeks postpartum (*n* ranged from 386–441 for individual CpG units). The maternal and infant characteristics within this subpopulation were representative of the whole population (data not shown). The association between postpartum alcohol consumption and *DRD4* methylation was found to be even stronger in the breastfeeding subpopulation (i.e. CpG.6:Δ4.01, 95%CI:1.91,6.11%, $P = 0.0002$; Supplementary Table S4).

Discussion

The strongest and most consistent finding from our study was an association between postpartum alcohol consumption and increased infant *DRD4* methylation (at 13 of 19 CpG units) assessed contemporaneously. Furthermore, postpartum alcohol consumption remained significantly associated with increased methylation, even after excluding women drinking during pregnancy, as well as controlling for other potential confounding factors. Our results also showed that this association was strongest among women who reported breastfeeding for the first 8-weeks postpartum, suggesting that transmission of alcohol through the breast milk may help account for this finding.

Dopaminergic Gene Methylation in Other Studies

DRD4 is enriched in the brain, primarily in the pituitary, amygdala, cerebral cortex and hypothalamus [26]. *DRD4* is a key component of dopamine signalling and was chosen for this candidate gene study due to the way alcohol effects dopamine signalling. It is a D2-like receptor that acts in postsynaptic signal transduction facilitating the inhibition of adenylate cyclase [21]. Alcohol does not directly interact with dopamine receptors such as *DRD4*; however, when alcohol is consumed, it causes dopamine release in the brain [27]. This in turn indirectly modulates dopaminergic signal transduction, activating reward pathways which can also lead to alcohol dependence [17]. Further, genetic variants in *DRD4* have been associated with attention deficit hyperactive disorder, alterations in inhibitory behaviour and other childhood behavioural problems [28].

No previous study has investigated the association between *DRD4* methylation and perinatal alcohol exposure, although other phenotypes have been explored. *DRD4* methylation in peripheral blood has been positively associated with the risk of schizophrenia in adult males ($n = 45$) but not females [29]. Findings of a study of monozygotic and dizygotic twins ($n = 182$), suggest that environmental, rather than genetic factors play a more important role in influencing DNA methylation of this gene in buccal cells [23]. Another study found increased blood *DRD4* promoter methylation in alcohol dependent adults versus controls of European American ethnicity ($\Delta + 0.9\% \pm 0.1$ SEM, $P = 0.0003$, $n = 249$) [22]. This is a similar effect size for mean methylation observed in our study with postpartum alcohol use and infant DNA methylation. Of note, the greatest effect size observed in our study was with CpG.6, and this lies close to a specific Protein-1 transcription factor binding site, which is 9 bp upstream [30]. This transcription binding factor is known to bind to CpG-rich genetic motifs within gene promoter regions, such as the one investigated within this study. Increased CpG.6 methylation in this region could block the binding of this transcription factor and thus suppress gene transcription [31], highlighting a potential functional role of differential methylation at this site. Although the observed effect sizes are relatively small, as is the case for most studies in this area, many small changes in DNA methylation across a network of genes, such as those within the dopaminergic pathway, may accumulate and result in potential significant detrimental effects [32].

DNA methylation levels of another gene involved in dopaminergic signalling, *DAT1*, has also been investigated in

Table 3. infant *DRD4* methylation according to maternal alcohol consumption at 8-weeks postpartum

CpG unit	Alcohol	*n*	Mean methylation (95%CI)	Difference (%)	*P*
1	No	304	80.4 (79.8,81.0)	+0.49	0.20
	Yes	494	80.9 (80.4,81.3)		
2_3	No	303	81.4 (80.8,81.2)	+0.79	0.029
	Yes	496	82.2 (81.8,82.6)		
4_5	No	311	38.7 (38.3,39.1)	+1.11	<0.0001
	Yes	502	39.8 (39.5,40.5)		
6	No	279	60.5 (59.3,61.7)	+3.20	0.0001
	Yes	441	63.7 (62.7,64.6)		
7	No	293	69.6 (68.6,70.6)	+2.00	0.0033
	Yes	477	71.6 (70.8,72.5)		
8	No	285	71.8 (70.9,72.8)	+1.76	0.0037
	Yes	475	73.6 (72.8,74.3)		
9	No	294	65.6 (64.5,66.7)	+1.39	0.037
	Yes	484	67.0 (66.2,67.7)		
12_13_14	No	312	28.2 (27.7,28.8)	+0.98	0.0050
	Yes	500	29.2 (28.8,29.6)		
15_16_17	No	296	22.0 (21.5,22.5)	+0.61	0.073
	Yes	465	22.6 (22.2,23.1)		
21_22_23	No	331	18.1 (17.7,18.6)	+0.69	0.028
	Yes	498	18.8 (18.4,19.2)		
24_25_26	No	312	31.5 (31.1,32.0)	+1.00	0.0011
	Yes	501	32.5 (32.2,32.9)		
27	No	310	33.4 (33.0,33.8)	+0.77	0.0010
	Yes	501	34.2 (33.9,34.5)		
28_29	No	311	18.5 (17.9,19.0)	+1.27	0.0003
	Yes	498	19.7 (19.3,20.5)		
30	No	301	24.2 (23.3,25.1)	+1.04	0.0530
	Yes	481	25.3 (24.6,25.9)		
32	No	299	1.9 (1.6,2.1)	−0.07	0.67
	Yes	486	1.8 (1.6,2.0)		
34_35	No	312	24.0 (23.6,24.4)	+0.52	0.041
	Yes	502	24.5 (24.2,24.8)		
36	No	306	13.1 (12.6,13.7)	+0.78	0.0300
	Yes	496	13.9 (13.5,14.4)		
37_38	No	311	8.3 (8.0,8.6)	+0.06	0.76
	Yes	501	8.4 (8.1,8.6)		
39	No	312	3.6 (3.4,3.7)	+0.21	0.070
	Yes	502	3.8 (3.6,3.9)		
Mean	No	202	36.5 (36.0,37.0)	+0.91	0.0049
	Yes	334	37.4 (37.0,37.8)		

association with alcohol exposure. *DAT1* is a dopamine transporter that regulates dopamine concentration and the duration of dopamine neurotransmission within neural synapses by actively transporting it from the synaptic terminal back into the cell [33]. Although *DAT1* is transcribed from a different genomic region (chr5), the transporter interacts with *DRD4* by making dopamine available across neuronal synapses for signal transduction. Only one study has investigated an association between prenatal alcohol use and infant DNA methylation within the *DAT1* promoter in cord blood ($n = 164$) [15]. This study found that in infants whose mothers binge-drank during pregnancy, there was an approximate 20% increase in *DAT1* promoter methylation when compared with mothers who were drinkers. However, no significant results were found when comparing promoter methylation between drinkers or binge-drinkers and non-drinkers. A few other studies of *DAT1* methylation investigated a direct association with alcohol exposure in adults. A study of 29 alcohol dependent Caucasian adult males found that relapsed patients ($n = 17$) had an average 3% higher *DAT1* methylation levels in blood compared with the abstinent group ($n = 12$) [34] but this did not reach significance ($P = 0.095$). In contrast, a larger study of 171 alcohol dependant adult Caucasian men found no significant difference in mean *DAT1* methylation levels in leucocytes, compared with 160 matched healthy controls [35]. However, at one of 23 CpG sites analysed, increased methylation was observed in the control group ($P = 0.029$, effect size not stated). Due to its integral part in dopaminergic signalling pathways and these initial findings, *DAT1* would be a candidate gene for further studies in maternal alcohol use and differential infant methylation.

Alcohol and Breastfeeding

Our primary finding of an association between maternal alcohol consumption early postpartum and infant *DRD4* methylation in buccal cells was unexpected and could be explained, at least in part, by the transmission of alcohol via breast milk. Ethanol, found in alcoholic drinks, is water soluble and passively diffuses into breast milk from the blood stream. Within an hour, alcohol levels in breast milk can reach the same as blood alcohol levels [36]. The vast majority of studies that have investigated maternal alcohol use and infant outcomes focus solely on the prenatal period; however, there is prior evidence to suggest that breast milk can modify DNA methylation and gene expression patterns. This could help explain the infant health benefits from breastfeeding (including lower risk of infections, obesity and related disorders). For example, lactoferrin in breast milk may reduce the inflammatory response by inhibiting NF-kappaB gene expression [37] and binding of lactoferrin to unmethylated CpGs in bacterial DNA in turn inhibits the immunostimulatory, hence inflammatory effects on human B cells [38, 39]. The high cholesterol content of breast milk may reduce endogenous cholesterol synthesis through downregulation of gene expression, which could explain the reduced cholesterol levels of adults who were breastfeed in infancy [37].

Our findings highlight the need to consider breastfeeding in cohort studies of infant DNA methylation patterns generally, as well as the amount of alcohol neonates are exposed to though breast milk consumption. This is especially important given that 62% of women have reported the consummation of alcohol in the early postnatal period, an important period of brain development [40].

Further Research

Although this initial analysis has investigated the associations between alcohol use at different stages of pregnancy and in the early postpartum, and infant *DRD4* methylation, it is possible that the dose of exposure during pregnancy is also important, and this is an avenue for future research in Triple B. Compared with heavy alcohol use, less is known about low or moderate maternal drinking pre or post birth. The majority of research concerns foetal alcohol spectrum disorder with its major phenotypic alterations as the primary focus. However, direct effects of low to moderate drinking may be too subtle to detect. A meta-analysis ($n = 11\,900$) of children aged 9 months to 5 years showed that moderate drinking during pregnancy was associated with poor child behaviour [41]. However, this meta-analysis only included 3 studies. Another study ($n = 4496$) found that low–moderate alcohol during pregnancy ($\leq$1 standard drink/week) was not associated with intrauterine growth

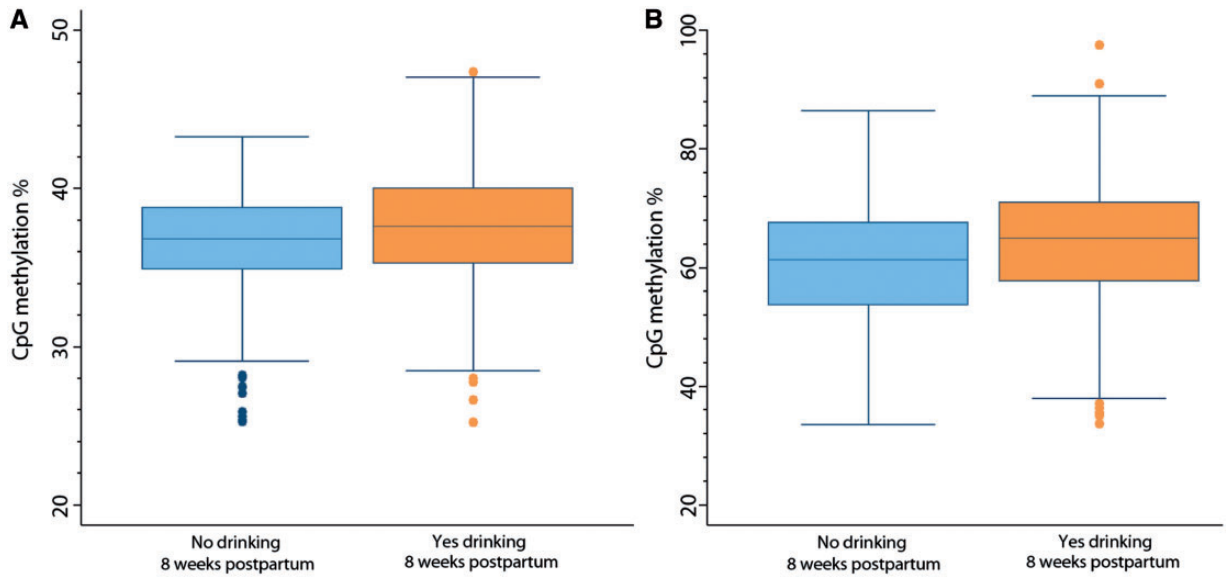

Figure 1. *DRD4* promoter methylation in infants according to maternal alcohol consumption in the first 8-weeks postpartum. (**A**) Mean promoter methylation (mean effect 0.91 95%CI:0.28,1.54, $P = 0.0049$). (**B**) CpG.6 methylation (mean effect 3.20, 95%CI:1.66,4.75%, $P = 0.0001$). Whiskers indicate 1.5*IQR, above and below the 75th and 25th percentile, respectively

Table 4. population characteristics of mothers and infants, according to alcohol use 8-weeks postpartum

Population characteristics	Alcohol consumption 8-weeks postpartum		
	No (n= 312)	Yes (n= 502)	P
Maternal characteristics		Mean ± SD	
Maternal age at birth (years)	31.9 ± 5.1	33.0 ± 4.9	<0.0029
		n(%)	
Country of birth:			
Australia	152 (51.3)	309 (61.6)	<0.001
Other	160 (48.7)	193 (38.4)	
Currently living with partner	285 (91.4)	472 (94.0)	0.146
Education level			
Year 12 or under	70 (22.4)	81 (16.1)	0.015
Completed Tafe/tech school	51 (16.4)	65 (13.0)	
Completed university/college	191 (61.2)	356 (70.9)	
Employment			
Full-time/self-employed	120 (38.5)	254 (50.6)	0.48
Part time/casual	60 (19.2)	93 (18.5)	
Home/student/unemployed	132 (42.3)	155 (30.9)	
Maternal tobacco use			
During pregnancy	52 (16.8)	86 (17.2)	0.88
8-weeks postpartum	25 (8.0)	52 (10.4)	0.27
Infant characteristics			
		Mean ± SD	
Gestation age at birth (weeks)	39.1 ± 1.9	39.5 ± 1.5	0.0039
Birthweight (kg)	3.4 ± 0.6	3.5 ± 0.5	0.040

restriction, preterm delivery or low birthweight, all reported outcomes of heavy pregnancy drinking [42]. Further research into DNA methylation effects from drinking, especially at low to moderate amounts of alcohol, and coupled with breastfeeding, is needed. Further, an important next step within this cohort will be to determine whether differential *DRD4* methylation is associated with developmental health outcomes in infants.

Strengths and Limitations

The major strength of this study is the large sample size combined with extensive data on the mother–infant dyads. This included detailed alcohol exposure data and a range of potential confounding factors. Thus, this study makes an important contribution to the field. Potential limitations to the current analysis relate to the *DRD4* methylation data obtained. Methylation is known to be dynamic, differing between tissues and cell types [43], thus the findings presented here are unique to buccal samples collected from infants at 8-weeks and cannot be generalized to other tissues or time-points. Even though neuronal genes were of specific interest in this study, buccal cells were used and preferred as a biological sample for their non-invasive collection method and possible usefulness as a biomarker. They are also a very pure cell population derived from

embryonic ectoderm, from which neural tissue also arises [44]. Further, recent data have highlighted the utility of buccal-derived DNA for studies of central nervous system related phenotypes [23].

As buccal cells would be in direct contact with breast milk, results may be influenced by maternal DNA contamination from the milk, resulting in erroneous results. A further limitation to this study is in that *DRD4* variable number tandem repeats were not accounted for, which have been shown to be associated with alcohol seeking behaviour [45] and could possibly influence differences in methylation values [46]. Dose response should be investigated in future studies, as well as other longitudinal time-points, to observe if these effects remain stable. Another limitation to consider is the small effect sizes observed, although this is within the range commonly observed in studies of early-life exposure [32, 47]. Although we do not yet know how these could translate into biological differences, it is possible that the accumulation of many small epigenetic changes during development could be sufficient to result in detrimental health outcomes [48]. Further EWASs will enable us to determine whether groups of genes linked to common signalling pathways are disrupted. Although we considered a range of covariates in our analysis, it remains possible that other confounders, not considered here, have influenced the associations. Finally, the fact that no buccal samples were collected at birth made it impossible to fully explore postnatal exposure in isolation, therefore where possible, these should be collected in future birth cohort studies.

Conclusion

The Australian Breastfeeding Association suggest that it is safe to have up to two standard drinks a day after the child is 1 month old, as long as the feed is before the alcohol consumption [49]. This study has found that alcohol exposure postpartum is associated with increased infant methylation. If further research shows that effects translate into infant health risks, redefinition of standards may be required, such as altering the alcohol free postnatal period and the suggested amount of alcohol which is consumed. Further studies are needed to replicate our findings, preferably ones that incorporate measurements of genetic variation into modelling of associations, as well as the timing and dose of exposure pre and postnatally. Only with robustly replicated findings, can the true reliability of these associations be ascertained, such as those consistently replicated studies that found smoking associated changes in methylation [47, 50]. Future studies could focus on the biological relevance of this methylation difference and how it is achieved, whether it equates to changes in gene expression, whether it remains stable over time and whether these differences are associated with infant health and development. The potential for epigenetic variation to mediate the relationship between maternal exposures and childhood outcomes is an important area of investigation, with great potential to influence the health of future generations through the development of novel interventions.

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Supplementary data

Supplementary data is available at EnvEpig online.

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