

The influence of prenatal alcohol exposure and maternal diet on offspring DNA methylation: a cross-species study

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Abstract

Alcohol consumption in pregnancy can affect genome regulation in the developing offspring but results have been contradictory. We employed a physiologically relevant murine model of short-term moderate prenatal alcohol exposure (PAE) resembling common patterns of alcohol consumption in pregnancy. Moderate early PAE was sufficient to affect site-specific DNA methylation in new-born pups without altering behavioural outcomes in adult littermates. Whole genome-bisulphite sequencing of neonatal brain and liver revealed stochastic influence on DNA methylation that was mostly tissue-specific, with some perturbations likely originating as early as gastrulation. Methylation changes were enriched in non-coding genomic regions with regulatory potential indicative of broad effects of alcohol on genome regulation. Replication studies in human cohorts with fetal alcohol spectrum disorder suggested some effects were metastable at genes linked to disease-relevant traits including facial morphology, intelligence, educational attainment, autism, and schizophrenia.

A maternal diet high in folate and choline protected against some of the damaging effects of PAE on DNA methylation. Our studies demonstrate that moderate early exposure is sufficient to affect fetal genome regulation even in the absence of overt phenotypic changes and highlight a role for preventative maternal dietary interventions.

eLife assessment

This **important** study examines the effects of prenatal alcohol exposure and maternal diet on offspring DNA methylation, revealing that alcohol can alter epigenetic patterns and impact brain and organ development in the fetus, with some changes preventable by a diet rich in folate and choline. The work identifies several differentially methylated regions linked to adverse health outcomes from alcohol exposure, but the evidence is somewhat **incomplete**, as the paper currently lacks comprehensive methodological details and sensitivity analyses. Further analysis of the functional relevance of these DNA methylation changes, particularly addressing the current technical and statistical shortcomings, would increase the study's novelty and significance.

Introduction

Alcohol consumption in pregnancy is the most common preventable cause of neurodevelopmental impairments in children (1 [↗](#)). Alcohol can pass through the placenta acting as a teratogen in fetal tissues causing physical, cognitive, behavioural, and neurodevelopmental impairment in children at high doses with lifelong consequences for health. Fetal Alcohol Spectrum Disorder (FASD) and Fetal Alcohol Syndrome (FAS) can arise at binge levels of exposure, although not always at lower levels of exposure. Whether PAE is sufficient to induce overt physiological abnormalities depends on multiple environmental and genetic factors including the dose and timing of alcohol use during pregnancy, maternal diet, smoking, stress, and potentially other factors that collectively influence fetal outcomes (2 [↗](#)–4 [↗](#)).

Patterns of alcohol consumption in pregnancy vary, but epidemiological surveys suggest most women in Western countries consume moderate to high levels between conception until recognition of pregnancy (5 [↗](#)), after which time consumption largely ceases, apart from occasional use (6 [↗](#)). While the effects of binge-levels of exposure are well documented to cause FASD, more subtle effects that reflect the more common patterns of drinking are unclear and more research is needed to support public health initiatives to reduce alcohol consumption in pregnancy.

Studies suggest alcohol can disrupt fetal gene regulation through epigenetic mechanisms. DNA methylation is one epigenetic mechanism involving the catalytic addition of methyl groups to cytosines bases within cytosine-guanine (CpG) dinucleotide motifs during one-carbon metabolism. Methylation of DNA can alter chromatin density and influence patterns of gene expression in a tissue-specific and developmentally appropriate manner and disruption to this process may cause of some of the difficulties experienced by people with FASD (7 [↗](#), 8 [↗](#)). Previous studies on human participants (9 [↗](#)–11 [↗](#)) and animals (12 [↗](#)–14 [↗](#)) report alcohol can disrupt DNA methylation either globally (11 [↗](#)–13 [↗](#)), and/or at specific gene regions (10 [↗](#), 13 [↗](#), 14 [↗](#)). Our recent systematic review however found limited replication of effects between studies suggesting the effects of alcohol on DNA methylation may be stochastic and influenced by numerous confounding factors (15 [↗](#)). PAE can either directly inhibit DNA methyltransferase enzymes or disrupt one-carbon metabolism via inhibition of bioavailability of dietary methyl donors, such as folate and

choline to the fetus (16 [↗](#)) (17 [↗](#)). Choline in particular has been explored in several clinical trials to reduce cognitive deficits caused by PAE in affected individuals (18 [↗](#), 19 [↗](#)), or when administered during pregnancy (20 [↗](#), 21 [↗](#)), with encouraging results.

Given the lack of clarity around the effects of typical patterns of alcohol consumption, which often do not cause observable phenotypes, we conducted an epigenome-wide association study of early moderate PAE in mice with replication in human cohorts. The study was a controlled intervention investigating the impact of PAE on offspring DNA methylation comparing exposed and unexposed mice, with an additional arm comparing the effect of alcohol exposure in the context of a high methyl donor maternal diet. The exposure period covers the equivalent of pre-conception up until the first trimester in humans when neurulation occurs, reflecting a typical situation in which women may consume alcohol up until pregnancy recognition (22 [↗](#)). The primary outcome of the study was changes in offspring DNA methylation and secondary outcomes of behavioural deficits across neurodevelopmental domains relevant to FASD were also examined. We employed whole genome bisulfite sequencing (WGBS) for unbiased assessment of the epigenome in newborn brain and liver, two target organs affected by ethanol (23 [↗](#)), to explore tissue-specificity of effects and to determine any ‘tissue agnostic’ effects which must have arisen prior to the germ-layers separating in early gastrulation.

Results

Comparison of prenatal characteristics across treatment groups

To investigate the effects of PAE and HMD on offspring DNA methylation and behavioural outcomes, we employed a murine model with four treatment groups (**Figure 1** [↗](#)). The trajectory of weight gain during pregnancy was similar across all treatment groups with some evidence of more rapid weight gain in the HMD groups in the last 2 days (Linear mixed effects regression model; H₂O-HMD: -2.282 ± 0.918 g, $P = 0.0177$; PAE-HMD: -1.656 ± 0.814 g, $P = 0.0493$; figure S1a), although the total amount of weight gained between days 1 and 17-19 was not significantly different between treatment groups by linear mixed effects regression (Figures S1a-b). The total amount of liquid consumed over the course of pregnancy was significantly lower in HMD dams by unpaired t-test (Figures S1b and S2c). There was no significant difference in the average litter size (6.525 ± 0.297 pups, figure S1c) and pup sex ratios (Figure S1d) between treatment groups by unpaired t-test.

Effects of PAE and HMD on behavioural outcomes in adult mice

Behavioural testing was carried out on adult mice from each study group. There was no evidence that PAE had a significant effect on any of behavioural outcomes tested (Figure S3). Mice exposed to HMD exhibited greater locomotor activity, in terms of distance travelled (**Figure 2** [↗](#)).

Effects of PAE on DNA methylation in newborn mice tissue

Whole-genome bisulfite sequencing (WGBS) of brain and liver samples from 16 newborn pups was performed to determine the effects of PAE on fetal DNA methylation. Global levels of DNA methylation stratified across different genomic features were preserved across treatment conditions, with no major differences in average methylation content between groups (Figure S4). To investigate region-specific effects of PAE on newborn DNA methylation, we conducted genome-wide testing comparing exposed and un-exposed mice on the normal chow. We identified 78 differentially methylated regions (DMRs) in the brain and 759 DMRs in the liver ($P < 0.05$ and $\Delta > 0.05$) from ~19,000,000 CpG sites tested after coverage filtering (**Figure 3a-b** [↗](#)). These regions were annotated to nearby genes using *annotatr* and are provided in Tables S1-2. Two of the DMRs overlapped in mouse brain and liver (tissue agnostic), but the remainder were tissue specific.

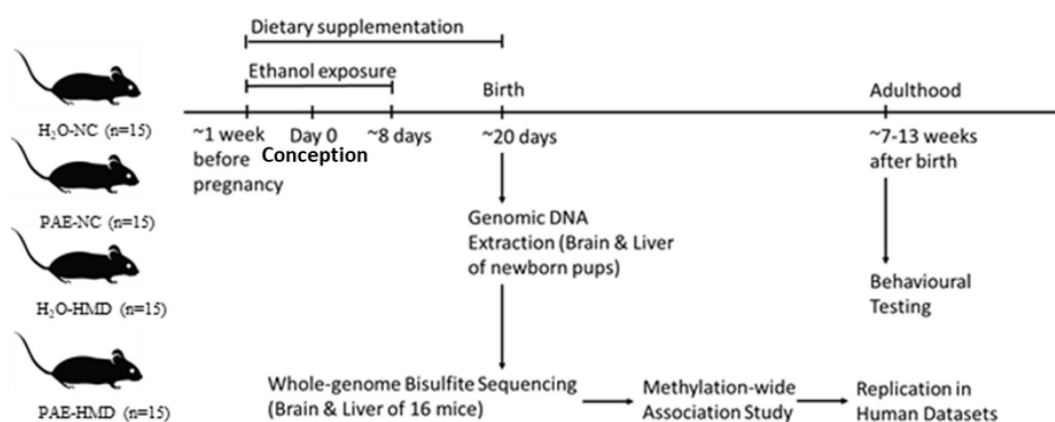


Figure 1.

Overview of PAE model.

A schematic representation of the experiment design is shown in [figure 1](#). Fifteen dams were allocated to each treatment group. Prenatal alcohol exposure (PAE) mice were exposed to ethanol (10% v/v in non-acidified reverse osmosis drinking water *ad libitum*) from one week before pregnancy to gestational days 8-10 and the remaining mice received water (H₂O). The PAE and H₂O groups received either normal chow (NC) or a high methyl donor (HMD) diet (NC containing 20 mg/kg folate and 4970 mg/kg choline) from one week before pregnancy until birth.

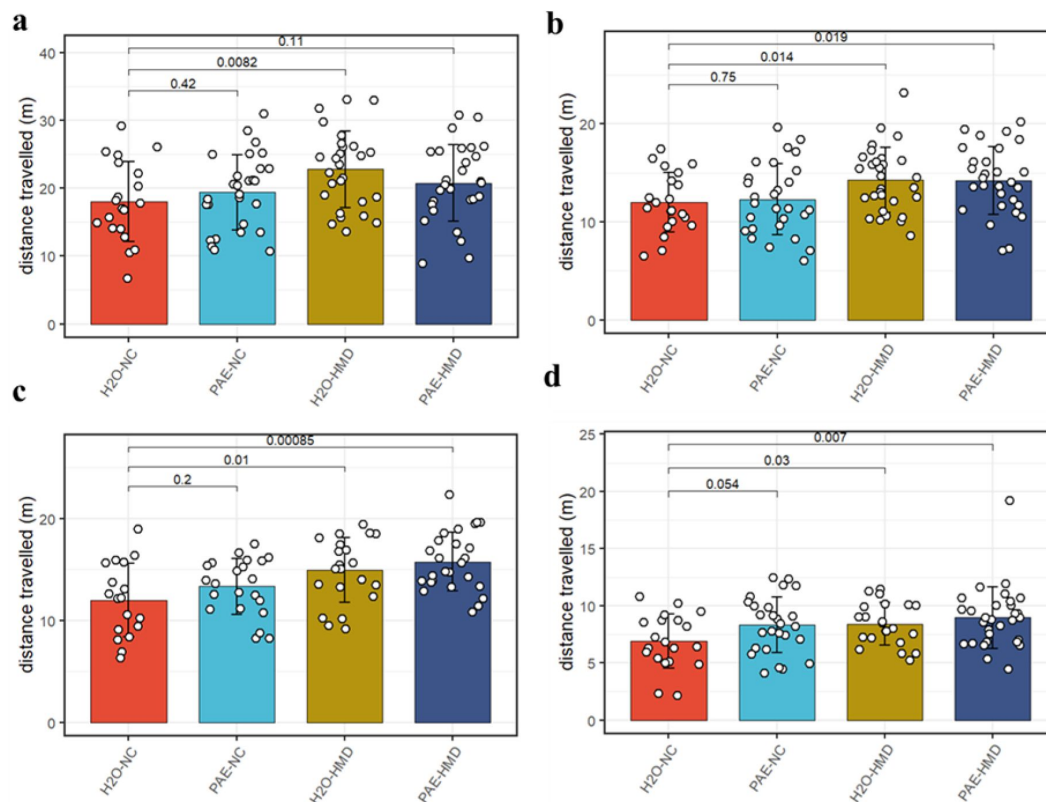


Figure 2.

HMD was associated with increased locomotor activity.

HMD was associated with increased locomotor activity compared to NC, indicated by significantly greater total distance travelled in the (a) open field test (sex $P = 0.0568$), (b) object recognition test (sex $P = 0.1519$), (c) elevated plus maze test (sex $P = 0.2043$), and (d) object in place test (sex $P = 0.1639$) by unpaired t-test. Bars show mean and standard deviation. Each point represents one mouse. NC = normal chow, HMD = high methyl diet, PAE = prenatal alcohol exposure. Time interval for each mouse was (a-c) 300 seconds and (d) 180 seconds. The p-value (Sex P) represents the statistical significance of the association between sex and the behavioral outcome. It was calculated using a two-way ANOVA that included PAE status and HMD status as factors, without considering any interactions.

Among these tissue agnostic regions was the *Impact* gene on chromosome 18, which was hypomethylated in PAE+NC mice compared to H₂O+NC mice in both the brain and liver (**Figure 3c-d**). The other tissue agnostic region was within 5kb downstream of *Bmf* and was hypermethylated in brain and liver tissue of PAE+NC mice.

In both tissues, hypomethylation with PAE in NC mice was more frequently observed in DMRs (52.6% of DMRs in brain, 93.5% of DMRs in liver hypomethylated with PAE) compared to hypermethylation. Some DMRs localised to the same genes in both brain and liver, although they were different regions. The three genes affected by PAE in both brain and the liver tissues were the Autism Susceptibility Gene 2 (*Auts2*), Androglobin (*Adgb*), and RNA Binding Protein Fox 1 (*Rbfox1*) genes (**Table 1**). In both brain and liver tissues, DMRs were enriched in non-coding intergenic and open sea regions and relatively underrepresented in coding and CpG island regions (**Figure 3e-f**). Using open chromatin assay and histone modification datasets from the ENCODE project, we found overwhelming enrichment ($p < 0.05$) of DMRs in open chromatin regions (ATAC-seq), enhancer regions (H3K4me1), and active gene promoter regions (H3K27ac), in mouse fetal forebrain tissue and fetal liver (**Table 2**). Gene ontology enrichment analysis of liver DMRs that did localise to genes showed enrichment in ten predominantly neuronal pathways, with neuron projection being the most significant (**Figure 3g**, Tables S3-4).

HMD mitigates the effects of PAE on DNA methylation

To determine whether administration of a HMD throughout pregnancy could mitigate the effects of PAE on offspring DNA methylation, we examined alcohol sensitive DMRs in the HMD mice. Compared to control mice, PAE+HMD mice exhibited methylation differences in only 12/78 (15%) brain (Table S7), and 124/759 (16%) liver (Table S8) DMRs, suggesting the effects were predominantly mitigated. Effect sizes compared to mice on the normal chow were substantially lower, in some cases more than 25% reduced in mice on the high methyl donor diet (**Figure 4**).

Replication studies in Human PAE and FASD case-control cohorts

We undertook validation studies in human cohorts to address the generalizability of our murine model of PAE. Only 36 of the 78 (46.2%) brain DMRs, and 294 of the 759 (38.8%) liver DMRs, had homologous regions in the human genome and were able to be tested. DNA methylation array data from 147 newborns buccal swabs from the Asking Questions About Alcohol in Pregnancy (AQUA) cohort (40) was available for analysis. We tested a total of 1,898 CpG sites that corresponded to mouse DMRs, comparing ‘never exposed’ newborns to ‘any exposure’ and found no evidence of differential methylation at these CpG (data not shown). We also accessed publicly available DNA methylation array measurements from buccal swabs taken from a Canadian clinical cohort of children with diagnosed FASD, and controls. To avoid confounding due to ancestry we analysed the 118 Caucasian individuals (30 FASD and 88 controls). Testing a total of 2,316 CpG sites that were homologous to mouse DMRs we statistically replicated 7 DMR associations with FASD status (FDR $P < 0.05$) after adjusting for participant age, sex, array number, and estimated cell counts (**Table 3**). Visual comparison of methylation changes across these seven DMRs revealed striking differences in effect sizes between people with FASD and mice (**Figure 5**). Genes associated with these DMRs are linked to clinically relevant traits in the GWAS catalogue including facial morphology (*GADD45A* (41)), educational attainment (*AP2B1* (42)), intelligence (*RP9* (43)), autism and schizophrenia (*ZNF823* (44)).

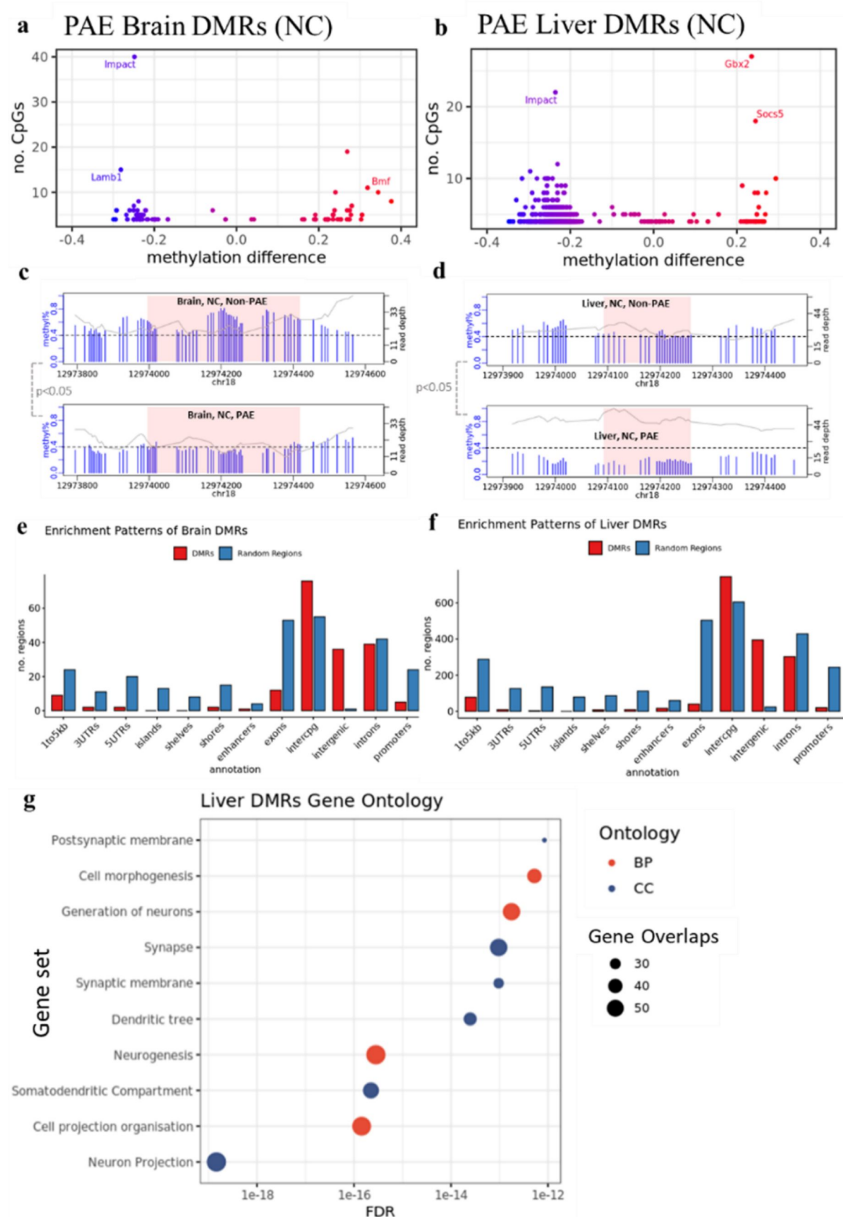


Figure 3.

PAE was associated with site-specific differences in offspring DNA methylation.

The majority of DMRs were hypomethylated with PAE in (a) brain and (b) liver of mice given normal chow. Each point represents one DMR. Point colour indicates change in DNA methylation with PAE. PAE was also associated with hypomethylation in the DMRs identified in the promoter of the *Impact* gene in (c) brain and (d) liver, within NC mice. Each plot represents a separate treatment group. Each blue vertical line indicates a CpG site, with the height and corresponding left y-axis indicating the methylation ratio. The grey line and corresponding right y-axis indicate coverage at each CpG site. The black horizontal dotted line indicates 40% methylation for comparison purposes. The x-axis indicates the base position on chromosome 18, with the pink shaded area highlighting the DMR. DMR plots include 200 base pair flanking regions on each side of the DMR. DMRs identified in (e) brain and (f) liver were enriched in intergenic and inter-CpG regions, whilst being underrepresented in CpG and gene regions. The bar plot compares the number of WGBS DMRs in red to a set of equivalent randomly generated regions in blue. (g) Gene ontology analysis of liver DMRs shows enrichment within neuronal cellular components and biological processes. BP/red point = biological process, CC/blue point = Cellular component. X-axis of point indicates FDR of ontology. Size of point indicates number of overlapping genes with ontology. There were insufficient number of DMRs identified in the brain for a gene ontology analysis.

Gene	Tissue	Intronic DMR	Δ meth	P-value
<i>Auts2</i>	Brain	chr5:131510296-131510465	-23.5%	<0.05
<i>Auts2</i>	Liver	chr5:131621828-131621999	-22.5%	<0.05
<i>Adgb</i>	Brain	chr10:10455557-10455883	-25.0%	<0.05
<i>Adgb</i>	Liver	chr10:10353338-10353613	-25.9%	<0.05
<i>Rbfox1</i>	Brain	chr16:6813039-6813217	-24.3%	<0.05
<i>Rbfox1</i>	Liver	chr16:6781985-6782330	-22.6%	<0.05

Table 1

Table of DMRs identified in the intronic regions of genes that contained DMRs in 561 both the brain and liver.

Δ meth indicates the percentage change in average methylation level 562 within the DMR with PAE compared to non-PAE mice.

Assay type	Brain DMRs	Brain randomised regions	Liver DMRs	Liver randomised regions
ATAC-seq	21/78 (26.92%) P = 0.01	1/78 (1.28%) P = 0.16	53/759 (6.98%) P = 0.01	22/759 (2.90%) P = 0.31
H3K4me1	4/78 (5.13%) P = 0.03	2/78 (2.56%) P = 0.18	38/759 (5.01%) P = 0.05	35/759 (4.61%) P = 0.32
H3K27ac	9/78 (11.54%) P = 0.01	2/78 (2.56%) P = 0.74	48/759 (6.32%) P = 0.01	19/759 (2.50%) P = 0.26

Table 2.

Number and percentage of brain and liver DMRs that overlap with tissue-specific regulatory regions.

ATAC-seq, H3K4me1 and H3K27ac regions were obtained at 0 days postnatal from the ENCODE database. P-values for permutation testing using a randomisation strategy.

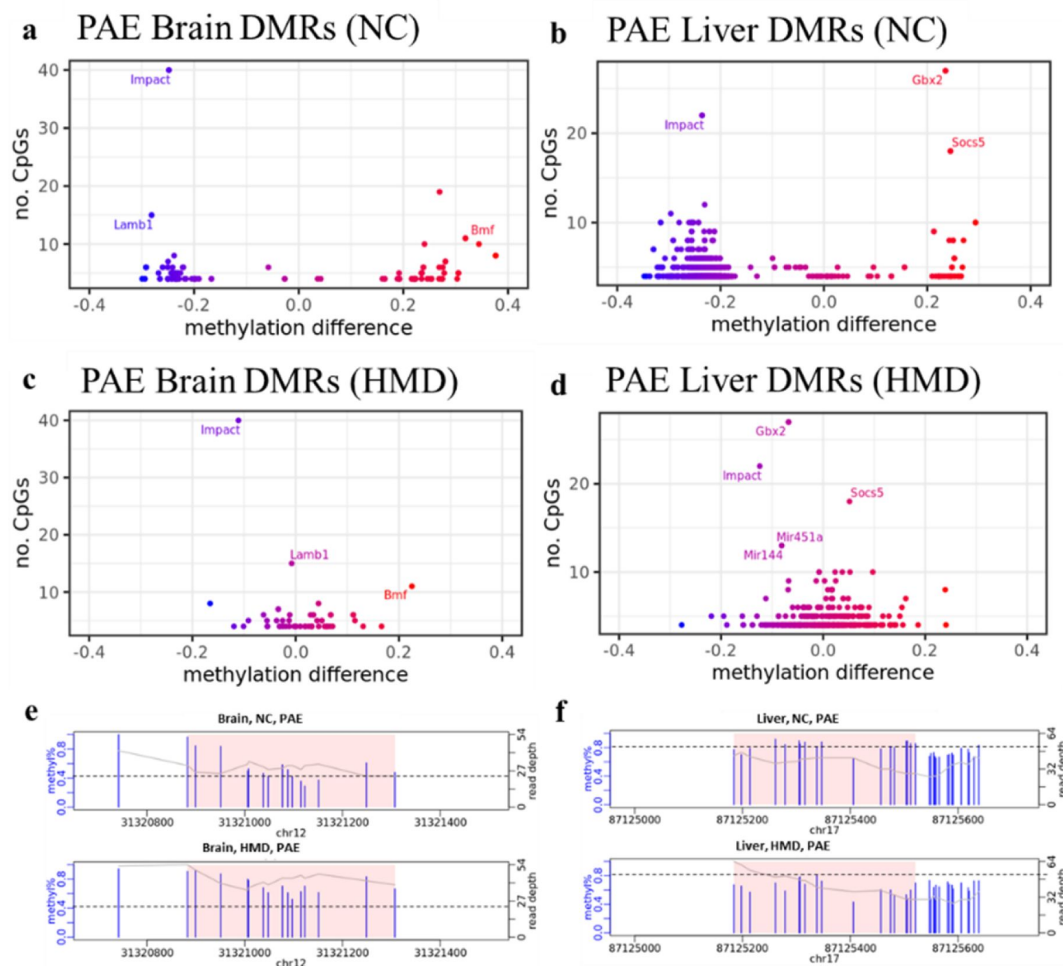


Figure 4.

HMD partially mitigated effects of PAE on offspring DNA methylation.

Average DNA methylation effect sizes above 30% with PAE were observed in some (a) brain, and (b) liver DMRs in NC mice. Mean absolute difference in methylation with PAE is reduced within the HMD mice in (c) brain, and (d) liver. Each point represents one DMR. Point colour indicates change in DNA methylation with PAE. Points with a high number of CpGs and methylation difference are annotated with associated gene if located within a genic region. HMD was associated with (e) hypermethylation in the DMR identified proximal to *Lamb1* on chromosome 12 in brain and (f) hypomethylation in the DMR identified proximal to *Socs5* on chromosome 17 in liver. Each plot represents a separate treatment group. Each blue vertical line indicates a CpG site, with the height and corresponding left y-axis indicating the methylation ratio. The grey line and corresponding right y-axis indicate coverage at each CpG site. The black horizontal line indicates (e) 40% and (f) 80% methylation for comparison purposes. The x-axis indicates the base position on the chromosome, with the pink shaded area highlighting the DMR. DMR plots include 200 base pair flanking regions on each side of the DMR.

DMR	organism	tissue	chr	start	end	width	no. cpgs	FDR	meandiff	gene
1	human	buccal	1	68151571	68152310	740	5	0.028636	-0.00497	<i>GADD45A</i>
2	human	buccal	19	13000782	13002357	1576	11	0.000197	-0.00203	<i>GCDH</i>
3	human	buccal	7	33148815	33149316	502	11	0.001149	-0.00011	<i>RP9</i>
4	human	buccal	17	33905444	33905888	445	14	0.000171	-0.00359	<i>AP2B1, PE X12</i>
5	human	buccal	17	27181503	27182342	840	11	0.018536	-0.00246	<i>ERAI1, FA M222B</i>
6	human	buccal	19	12992181	12992479	299	9	0.037431	-0.00179	<i>CTD- 2265O21.7, DNASE2</i>
7	human	buccal	19	11849531	11850013	483	9	0.022724	-0.00244	<i>ZNF823</i>
1	mouse	liver	6	67034885	67035082	197	4	<0.05	-0.220833	<i>E230016M1 IRik</i>
2	mouse	liver	8	84901298	84901544	246	5	<0.05	-0.234457	<i>Klfl</i>
3	mouse	liver	9	22453836	22453893	57	5	<0.05	-0.226427	<i>Rp9</i>
4	mouse	brain	14	21403570	21403622	52	4	<0.05	-0.234193	<i>Adk</i>
5	mouse	liver	11	78069463	78070002	539	9	<0.05	-0.255864	<i>Mir144, Mir451a</i>
6	mouse	liver	11	78072079	78072313	234	4	<0.05	-0.215227	<i>Mir144, Mir451a</i>
7	mouse	liver	2	177091927	177092945	1018	5	<0.05	-0.224354	Intergenic

Table 3.

DMRs identified in the murine model that were validated in the Lussier et al. human case-control cohort for a clinical diagnosis of FASD.

The upper section describes properties of Lussier et al. human DMRs. The lower section describes properties of the equivalent murine model DMRs.

Candidate Gene Analysis of previously defined alcohol sensitive regions

In candidate gene studies there were 21 CpG sites ($FDR < 0.05$) identified in the brain from 15,132 CpG sites tested, including two sites in the *Mest* (*Peg1*) gene and 19 sites in *Kcnq1* (*KvDMR1*) (Table S9). There were nine FDR-significant CpG sites identified in the liver out of 15,382 CpG sites tested, all of which were in *Peg3* (Table S10). All FDR-significant CpG sites identified in both tissues were hypomethylated in mice with PAE.

Discussion

In this study, found that moderate early (first trimester) PAE was sufficient to induce site-specific differences to DNA methylation in newborn pups without causing overt behavioural outcomes in adult mice. Global levels of DNA methylation were not significantly different with PAE, and effects were characterized predominantly by a loss of methylation (hypomethylation), mostly at non-coding regions of the genome. In our model, alcohol's effects on DNA methylation were predominantly tissue-specific, with only two genomic regions and four genes that were similarly affected in both tissues. These perturbations must have arisen before the germ layers separated suggesting alcohol can perturb methylation events as early as gastrulation. In general, DMRs were enriched in non-coding regions of the genome with regulatory potential suggesting alcohol may have broad effects on genome regulation.

Both the human validation studies and the candidate gene analysis provide validity to our model for recapitulating some of the genomic disturbances reported in patients with clinical FASD. It is remarkable that associations were reproduced despite differences in biosamples and species and suggests that at least some methylation changes are stable over time. We replicated associations from published reports of hypomethylation within *Peg3* and *KvDMR1* from South African children with fetal alcohol syndrome (10). Both these genes are methylated in a parent-of-origin specific manner, suggesting that alcohol may affect imprinting processes, although results are not entirely consistent (45, 46). On the balance of this, we speculate duration of exposure, dose, and other tissue-related factors all likely influence the extent to which genome-regulation is perturbed and manifests as differences in DNA methylation.

Our results are encouraging for biomarker studies and aid in the prioritisation of associations for future follow-up, particularly in relation to diagnosis of FASD. For example, three genes are zinc finger proteins (*RP9*, *PEX12*, and *ZNF823*) that play an important role in fetal gene regulation. Notably, *PEX12* is associated with Zellweger syndrome, which is a rare peroxisome biogenesis disorder (the most severe variant of Peroxisome biogenesis disorder spectrum), characterized by neuronal migration defects in the brain, dysmorphic craniofacial features, profound hypotonia, neonatal seizures, and liver dysfunction (47).

Another key finding from this study was that HMD mitigated some of the effects of PAE on DNA methylation. Preclinical studies of choline supplementation in rodent models have similarly reported attenuation of memory and behavioural deficits associated with PAE (48, 49), and mitigating effects on DNA methylation (50). These data have been largely consistent and collectively support the notion that alcohol induced perturbation of epigenetic regulation may occur, at least in part, through disruption of the one-carbon metabolism. The most encouraging aspect of this relates to the potential utility for evidence-informed recommendations for dietary advice or supplementation, particularly in population groups with limited access to antenatal care or healthy food choices.

Strengths of this study include the use of controlled interventions coupled with comprehensive assessment of the effects of PAE on multiple tissues. Caveats include a limited ability to determine the contribution of specific cell types within tissues to the methylation differences observed, and we did not assess markers of brain or liver physiology.

In conclusion, this study demonstrates that early moderate PAE can disturb fetal gene regulation and supports current public health advice that alcohol consumption during pregnancy, even at low doses, may be harmful.

Materials and Methods

Murine subjects and housing

To study the effects of PAE on offspring DNA methylation processes, we adapted a murine model study design that has previously reported DNA methylation changes at the *A^{Vy}* locus in Agouti mice (24) (Figure 1). This study received animal ethics approval from the Telethon Kids Institute Animal Ethics Committee (Approval Number: 344). Sixty nulliparous C57BL/6J female mice aged ~8 weeks were mated with equivalent stud male mice. Pregnant dams were randomly assigned to one of four treatment groups (n = 15 dams per group) that varied based on composition of the drinking water and chow given to the dams:

1. PAE-NC (Prenatal Alcohol Exposure-Normal Chow): 10% (v/v) ethanol in non-acidified water *ad libitum* from 10 days prior to mating until gestational days (GD) 8-10. This is intended to replicate typical patterns of drinking during the first trimester of pregnancy in humans. Dams received non-acidified reverse osmosis water for the remainder of pregnancy and normal chow (Rat and Mouse Cubes, Speciality Feeds, Glen Forrest, Australia) throughout pregnancy.
2. PAE-HMD (Prenatal Alcohol Exposure-High Methyl Donor diet): 10% (v/v) ethanol in non-acidified water *ad libitum* from 10 days prior to mating until GD8-10 and non-acidified reverse osmosis water for remainder of pregnancy. Isocaloric high methyl donor (HMD) chow consisting of 20 mg/kg folate and 4,970 mg/kg choline throughout pregnancy (Speciality Feeds, Glen Forrest, Australia).
3. H₂O-NC (Water-Normal Chow): non-acidified water and normal chow throughout pregnancy.
4. H₂O-HMD (Water-High Methyl Donor diet): non-acidified water and HMD chow throughout pregnancy.

Whole-genome bisulfite sequencing of newborn mouse tissues

Pups selected for WGBS in each intervention group were matched on sex and litter size to minimize variability in exposure. Two male and two female pups per treatment group (n = 16 total) were euthanised by intraperitoneal injection with ketamine and xylazine on the day of birth for WGBS of their brain and liver tissues. Mouse tissue samples were stored at -80°C. Remaining littermates grew until adulthood for behavioural testing. Ten milligrams of tissue were collected from each liver and brain. Total nucleic acid was extracted from the tissues using the Chemagic 360 instrument (PerkinElmer) and quantified with Qubit DNA High Sensitivity Kit (Catalogue Number: Q32854, Thermo Scientific). 100 ng of genomic DNA was spiked with 0.5 ng of unmethylated lambda DNA (Catalogue Number: D1521, Promega) to assess the bisulfite conversion efficiency. Each sample was digested with 2 µl RNase A (Invitrogen) at 37°C for 20 minutes to remove RNA. 100 ng of genomic DNA from each sample was sheared using a Covaris M220 (300bp settings, Covaris). Libraries were prepared using the Lucigen NxSeq AmpFREE Low DNA Library Kit (Catalogue Number: 14000-1, Lucigen), according to the manufacturer's instructions. Nextflex

bisulfite-seq barcodes (Catalogue Number: Nova-511913, PerkinElmer) were used as the adapters with incubation at 25°C for 30 minutes. The libraries were bisulfite converted using the Zymo EZ DNA Methylation-Gold Kit (Catalogue Number: D5005, Zymo Research) and PCR amplified using the KAPA HiFi Uracil PCR Kit (Catalogue Number: ROC-07959052001, Kapa Biosystems). The final libraries were assessed with the Agilent 2200 Tapestation System using D1000 Kit (Catalogue Number: 5067-5582). WGBS was performed by Genomics WA sequencing core on a NovaSeq 6000 (Illumina) using 2×150bp chemistry on an S4 flow cell. The bisulfite conversion rate in each tissue sample was at least 99%. The overall mean coverage in each sample was 9.69× (range: 6.51-12.12×).

Behavioural testing in adult mice

Littermates who were not sacrificed at birth were reared on normal chow and drinking water *ad libitum* until adulthood (~8 weeks after birth) when they underwent behavioural tests assessing five neurodevelopmental domains that can be affected by PAE including locomotor activity, anxiety, spatial recognition, memory, motor coordination and balance. These tests included the open field test (locomotor activity, anxiety) (25 [↗](#)), object recognition test (locomotor activity, spatial recognition) (26 [↗](#)), object in place test (locomotor activity, spatial recognition) (27 [↗](#)), elevated plus maze test (locomotor activity, anxiety) (28 [↗](#)), and two trials of the rotarod test (motor coordination, balance) (29 [↗](#)). Between mouse subjects, behavioural testing equipment was cleaned with 70% ethanol. Video recording was employed for all behavioural tests, except for the rotarod, and the assessment process was semi-automated using ANY-maze software (Stoelting Co., Wood Dale, Illinois, U.S.A.).

Statistical analysis

Dam characteristics and pup behavioural testing results were generally assessed using unpaired t-tests comparing each treatment group to the baseline control group that was given non-acidified reverse osmosis water and normal chow throughout pregnancy. Trajectories of liquid consumption and weight gain across pregnancy, which were assessed using a quadratic mixed effects model and the trajectory of chow consumption across pregnancy which was assessed using a linear mixed effects model. Raw fastq files were mapped to the mm10 mouse reference genome with BSseeker 2 (version 2.1.8) (30 [↗](#)) and CG-maptools (version number 0.1.2) (31 [↗](#)) using a custom bioinformatics pipeline. CGmap output files were combined as a bsseq object in the R statistical environment (32 [↗](#)). We filtered the X chromosomal reads and then combined reads from mice in the same treatment group using the *collapseBSseq* function, to maximise coverage prior to differential methylation analysis. CpG sites with an aggregated coverage below 10× in each tissue type were removed prior to modelling to ensure there was sufficient coverage in all assessed CpG sites. This retained 94.9% of CpG sites in the brain and 93.8% of CpG sites in the liver. Differentially methylated regions (DMRs) were identified within each tissue using a Bayesian hierarchical model comparing exposed and unexposed groups using the Wald test with smoothing, implemented in the R package *DSS* (33 [↗](#)). We declared DMRs as those with P-value < 0.05 based on the p-values of each individual CpG site in the DMR, and an effect size (delta) > 0.05. Gene ontology analysis was performed on the brain and liver DMRs using the Gene Set Enrichment Analysis computational method (34 [↗](#)) to determine if the DMRs were associated with any transcription start sites or biological processes. Brain and liver DMRs were tested for enrichment within ENCODE Project data sets (35 [↗](#)) by an overlap permutation test with 100 permutations using the *regioneR* package. The ENCODE Project data sets that were assessed included ENCFF845WSI, ENCFF764NTQ, ENCFF937JHP, ENCFF269TLO, ENCFF676TSV and ENCFF290MLR. DMRs were then tested for enrichment within specific genic and CpG regions of the mouse genome, compared to a randomly generated set of regions in the mouse genome generated with *resampleRegions* in *regioneR*, with equivalent means and standard deviations. We compiled a set of key genes and genomic regions identified in previous mammalian PAE studies for site-specific testing based on our prior systematic review of the literature (15 [↗](#)), which identified 37 candidate

genes (Tables S5-6). The brain and liver datasets were filtered to candidate gene regions and differential testing was then performed across the entire coding sequence, separately in the brain and liver of the mice on a normal diet using the *callDML* feature in DSS.

Validation studies in human cohorts

Validation studies in human cohorts with existing genome-wide DNA methylation data sets and matching PAE data are described in the Supplementary Material. Briefly, Illumina Human Methylation array. iDAT files were pre-processed using the *minfi* package (36) from the Bioconductor project (<http://www.bioconductor.org>) in the R statistical environment (<http://cran.r-project.org/>, version 4.2.2). Sample quality was assessed using control probes on the array. Between-array normalization was performed using the stratified quantile method to correct for Type 1 and Type 2 probe bias. Probes exhibiting a *P*-detection call rate of >0.01 in one or more samples were removed prior to analysis. Probes containing SNPs at the single base extension site, or at the CpG assay site were removed, as were probes measuring non-CpG loci (32,445 probes). Probes reported to have off-target effects in McCartney et al. (37) were also removed. Mouse DMRs were converted into human equivalent regions using an mm10 to hg19 genome conversion with the liftover tool in the UCSC Genome Browser (38). A minimum 0.1 ratio of bases that must remap was specified as recommended for liftover between regions from different species and multiple output regions were allowed. Differential testing of candidate mouse DMRs was carried out using the R package *DMRcate* (39) for each dataset and DMRs were declared as minimum smoothed false-discovery rate (FDR) < 0.05.

Data Availability Statement

The mouse whole-genome bisulfite sequencing data will be available on the GEO repository at <http://ncbi.nlm.nih.gov/geo>. The human array data used in the validation study is not publicly available due to privacy and ethical restrictions.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

D. Martino, M. Symons, A. Larcombe, R. Lister, D. Hutchinson, E. Muggli, J. Craig, J. Halliday, J. Fitzpatrick, S. Buckberry, M. Bestry and E. Elliott contributed to the study design and funding application. E. Chivers, A. Larcombe performed the mouse work including administering the mating, treatments, measurements, monitoring and extracting biological samples. E. Chivers, A. Larcombe and M. Bestry performed the mouse behavioural testing. C. Bakker analysed the videos from the mouse behavioural testing. M. Bestry prepared the whole-genome bisulfite sequencing libraries and performed the data analyses. N. Kresoje assisted with preparing whole-genome bisulfite sequencing libraries. S. Buckberry and D. Martino provided advice and support on the data analysis. J. Halliday and E. Muggli contributed human datasets for reproducibility analysis. M. Bestry and D. Martino drafted the manuscript. M. Symons, A. Larcombe, R. Lister, D. Hutchinson, E. Muggli, J. Craig, J. Halliday, J. Fitzpatrick, S. Buckberry, E. Elliott and N. Kresoje contributed to the development and editing of the manuscript.

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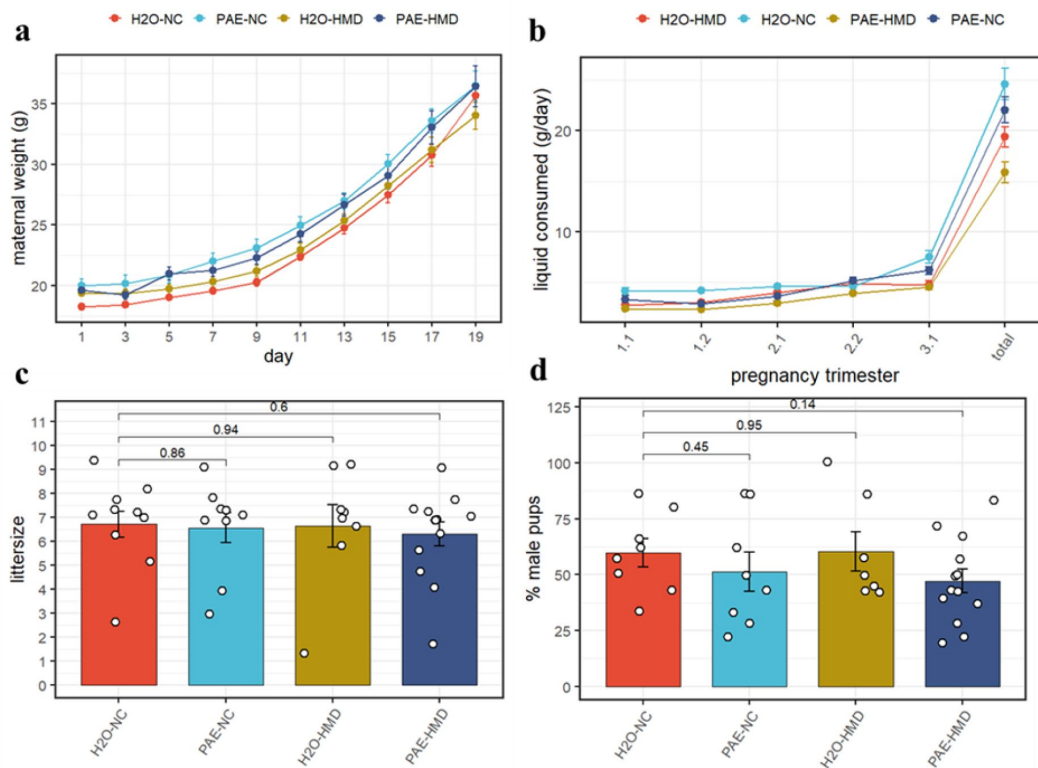


Figure S1.

PAE and HMD had varied effects on dam characteristics.

(a) Dam weight progression was significantly affected by HMD but not PAE by quadratic mixed-effects model without interaction (b) Trajectory of liquid consumption across pregnancy was affected by PAE and HMD by quadratic mixed effects model. PAE and HMD significantly interacted with trimester of pregnancy. (c) litter size and (d) pup sex ratios were not significantly associated with PAE or HMD by unpaired t-test or ANOVA. All line and bar plots show mean and standard deviation. NC = normal chow, HMD = high methyl diet, PAE = prenatal alcohol exposure. Comparisons show p-value by unpaired t-test compared to the H₂O-NC baseline treatment group.

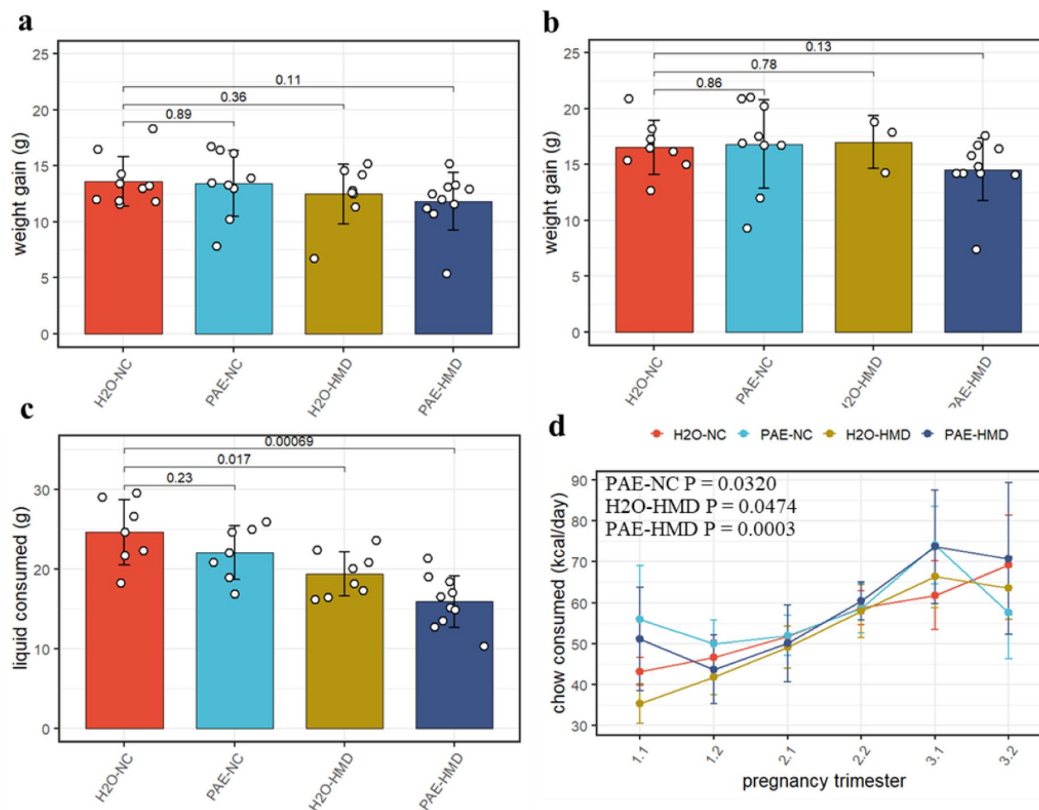


Figure S2.

PAE and HMD had varied effects on dam characteristics.

There was no significant difference in the average gain of weight in dams between (a) days 1-17 or (b) days 1-19 by treatment group. Both timepoints were included due to some pregnancies ending by day 19. (c) Mice given supplemented chow consumed significantly lower total quantity of liquid across pregnancy. Bar plots show mean and standard deviation for each treatment group. Each point represents one dam. (d) the trajectory of chow consumed across pregnancy significantly varied based on treatment group. Points show mean and standard deviation for each treatment group.

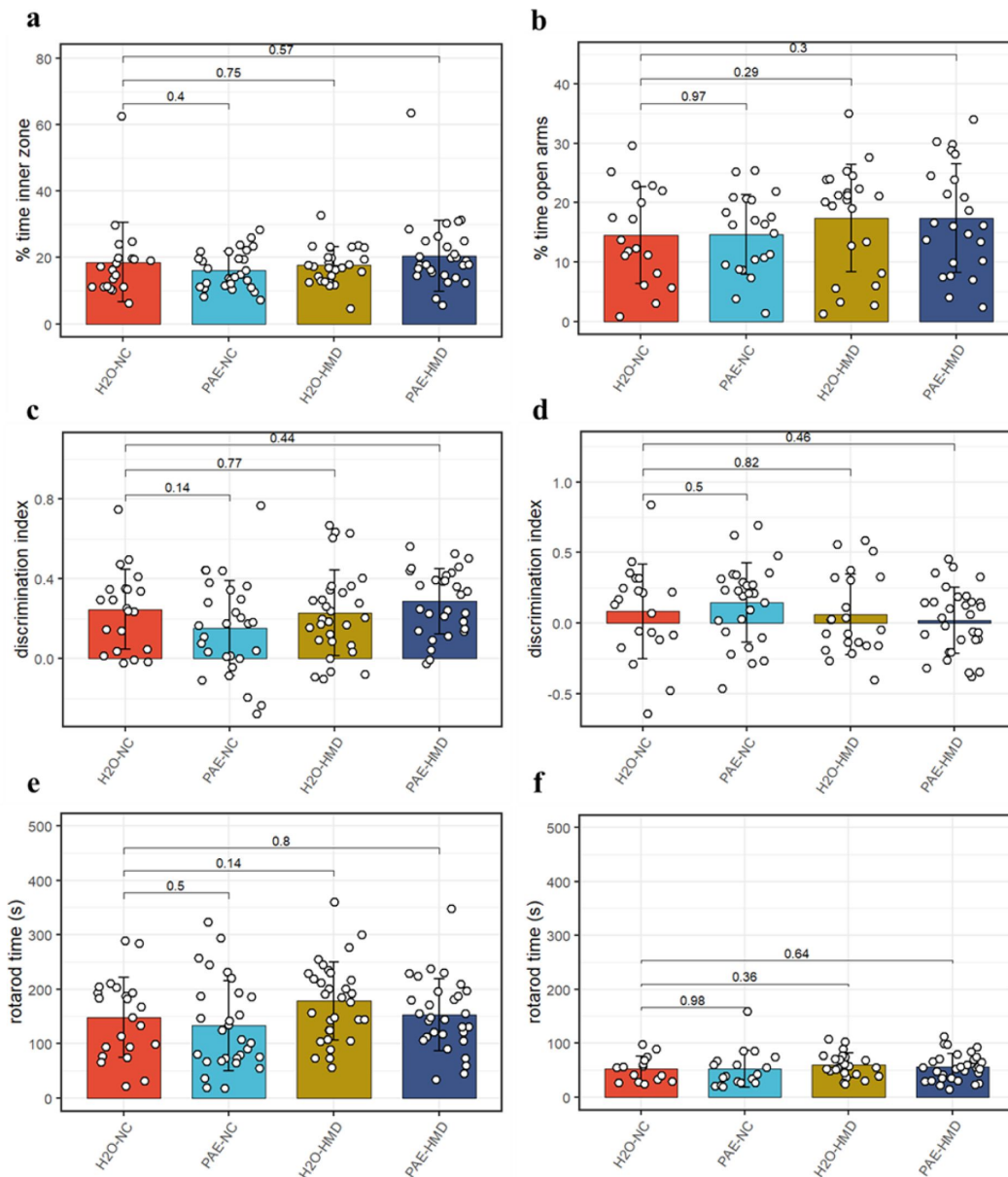


Figure S3.

PAE had no significant effect on other assessed behavioural outcomes.

PAE and HMD had no significant effect on anxiety as evident by no significant difference by unpaired t-test in the (a) percent time in the inner zone in the open field test (sex $P = 0.915$) and (b) percent time open arms in the elevated plus maze test (sex $P = 0.949$). PAE and HMD had no significant effect on spatial recognition as evident by no significant difference by unpaired t-test in the discrimination index in (c) object recognition (sex $P = 0.396$) and (d) object in place test (sex $P = 0.987$). PAE and HMD had no significant effect on motor co-ordination and balance as evident by no significant difference by unpaired t-test in times in (e) first rotarod test (sex $P = 0.634$) and (f) second rotarod test (sex $P = 0.143$). Bars show mean and standard deviation. Each point represents one mouse. NC = normal chow, HMD = high methyl diet, PAE = prenatal alcohol exposure. Time interval for each mouse was (a-c) 300 seconds and (d) 180 seconds. The p-value (Sex P) represents the statistical significance of the association between sex and the behavioral outcome. It was calculated using a two-way ANOVA that included PAE status and HMD status as factors, without considering any interactions.

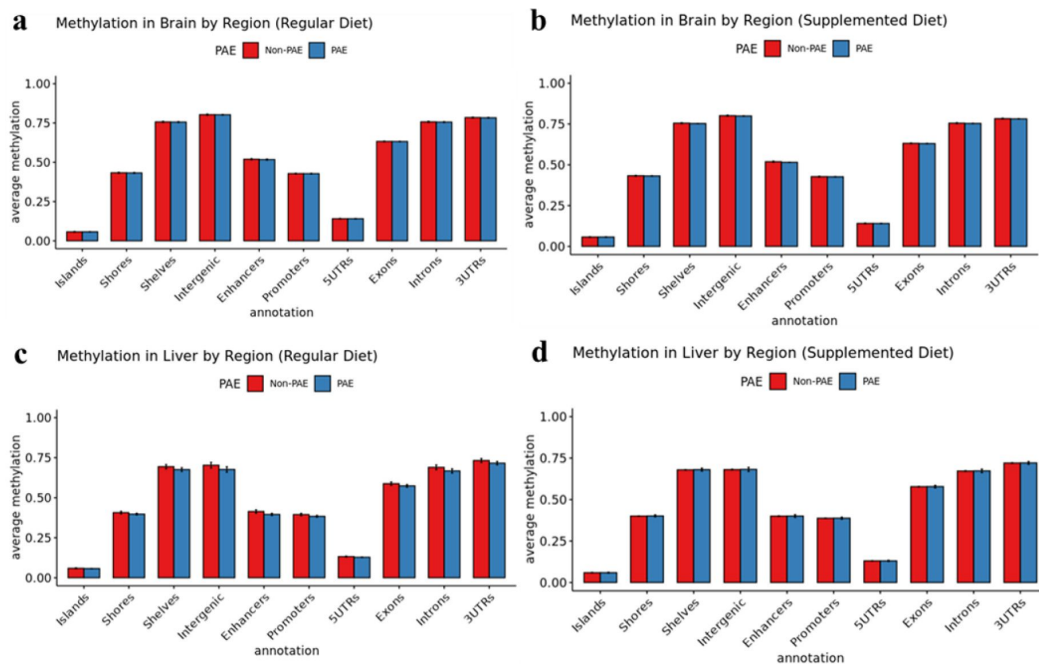


Figure S4.

Global DNA methylation boxplots.

(a) Across CpG sites that had at least 1X coverage in each sample, DNA methylation was significantly hypomethylated within brain DMRs located in all genic and CpG regions except for intergenic region and 3UTRs, which were significantly hypermethylated from PAE. (b) The equivalent analysis in liver showed significant hypomethylation of DMRs located in all genic regions except for CpG shelves. No significant results were identified within the equivalent analysis of (c) brain or (d) liver in mice given supplemented chow.

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Editors

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Reviewer #1 (Public Review):**Summary:**

This manuscript examined the impact of prenatal alcohol exposure on genome-wide DNA methylation in the brain and liver, comparing ethanol-exposed mice to unexposed controls. They also investigated whether a high-methyl diet (HMD) could prevent the DNA methylation alterations caused by alcohol. Using bisulfite sequencing (n=4 per group), they identified 78 alcohol-associated differentially methylated regions (DMRs) in the brain and 759 DMRs in the liver, of which 85% and 84% were mitigated by the HMD group, respectively. The authors further validated 7 DMRs in humans using previously published data from a Canadian cohort of children with FASD.

Overall, the findings from this study provide new insight into the impact of prenatal alcohol exposure, while also showing evidence for methyl-rich diets as an intervention to prevent the effects of alcohol on the epigenome. However, several methodological concerns limit the robustness of these results and should be addressed to further strengthen the conclusions of this study and its applicability to broader settings.

Strengths:

- The use of whole genome bisulfite sequencing allowed for the interrogation of the entire DNA methylome and DMR analysis, rather than a subset of CpGs.
- The combination of data from animal models and humans allowed the authors to make stronger inferences regarding their findings.
- The authors investigated a potential mechanism (high methyl diet) to buffer against the effects of prenatal alcohol exposure, which increases the relevance and applicability of this research.

Weaknesses:

- Mistakes and discontinuities in the reporting of results and methods made the manuscript difficult to follow. There was also some overuse of causal language and overinterpretation of

differences.

- The authors provide insufficient details to replicate their analyses, particularly for data quality control steps and statistical analyses.
- The sample size was very small for the epigenetic analyses, which limits the robustness of the findings. This limitation is further emphasized by the cutoffs used to identify DMRs, which did not include multiple test corrections and used a delta cutoff that was not supported by the sequencing depth.
- The authors do not account for potential confounders in their analyses, including birthweight, alcohol levels, and sex. This is a particular problem for the high-methyl diet analyses, in which the alcohol-exposed mice seemed to consume less alcohol than their non-diet counterparts.

Reviewer #2 (Public Review):

Summary:

Bestry et al. investigated the effects of prenatal alcohol exposure (PAE) and high methyl donor diet (HMD) on offspring DNA methylation and behavioral outcomes using a mouse model that mimics common patterns of alcohol consumption in pregnancy in humans. The researchers employed whole-genome bisulfite sequencing (WGBS) for unbiased assessment of the epigenome in the newborn brain and liver, two organs affected by ethanol, to explore tissue-specific effects and to determine any "tissue-agnostic" effects that may have arisen prior to the germ-layer commitment during early gastrulation. The authors found that PAE induces measurable changes in offspring DNA methylation. DNA methylation changes induced by PAE coincide with non-coding regions, including enhancers and promoters, with the potential to regulate gene expression. Though the majority of the alcohol-sensitive differentially methylated regions (DMRs) were not conserved in humans, the ones that were conserved were associated with clinically relevant traits such as facial morphology, educational attainment, intelligence, autism, and schizophrenia. Finally, the study provides evidence that maternal dietary support with methyl donors alleviates the effects of PAE on DNA methylation, suggesting a potential prenatal care option.

Strengths:

The strengths of the study include the use of a mouse model where confounding factors such as genetic background and diet can be well controlled. The study performed whole-genome bisulfite sequencing which allows a comprehensive analysis of the effects of PAE on DNA methylation. However, some weaknesses and limitations of the study are detected.

Weaknesses:

1. The low generalizability between mouse and human data alerts the validity of the mouse model designed in the study. On the same note, the authors failed to detect any significant effect on PAE-induced behavioral outcomes. I recognize that it is difficult to model all possible conditions of PAE in mice because the amount, frequency, and duration of alcohol consumption in humans vary significantly. Therefore, if the authors only focus on moderate PAE, it should be emphasized in the title and throughout the paper to avoid misinterpretation. In addition, is it possible to stratify the human data based on the level of PAE and compare it to the mouse data?
2. A major finding of the study is that PAE affects non-coding genomic regions in mice including enhancers and promoters. To improve the significance of the study, the authors need to back up this finding with transcriptome analysis and determine if these DMRs indeed affect gene expression.
3. The low generalizability between mouse and human data suggests that the regions affected by PAE may be species-specific. It is critical to analyze if PAE-induced DMRs in humans are also enriched in non-coding genomic regions. Considering the huge difference between mouse and human development, particularly in the brain, it is not surprising that different genomic loci are affected, but the affected loci may share similar features.

4. The specific brain regions and the lobes of the liver where the samples were taken should be clearly indicated.

5. I don't fully agree with the authors' interpretation that the two shared genomic regions affected in the brain and the liver "must have arisen before the germ layers separated". To claim so, the authors need to exclude the possibility that the two regions are just a coincidence due to the stochastic effect of PAE on DNA methylation.