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

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Abstract

Prenatal alcohol exposure (PAE) is associated with changes to offspring DNA methylation and adverse neurodevelopmental outcomes. Prior studies have utilised candidate gene or microarray approaches resulting in biased representation of the methylome. We employed an established experimental murine model of moderate PAE until gestational days 8-10, with genome-wide DNA methylation sequencing of neonatal brain and liver. Although global DNA methylation levels were generally preserved, we detected 78 alcohol-sensitive regions in neonatal brain, and 759 alcohol-sensitive regions in neonatal liver ($p < 0.05$ and delta beta > 0.05). Affected coding regions were significantly enriched in genes involved in

neurodevelopmental pathways, but behavioural outcomes were unaltered in adult littermates. Seven alcohol-sensitive regions identified in mice were statistically replicated in a human cohort of adolescents with fetal alcohol spectrum disorder (FASD) and known to be linked with traits related to facial morphology, intelligence, educational attainment, autism, schizophrenia, ageing and haematological disorders in the GWAS catalogue. The high methyl donor diet (HMD) across pregnancy partially protected against the effects of alcohol on DNA methylation in offspring tissues. Collectively this study provides a comprehensive evaluation of PAE on offspring DNA methylation and suggests prenatal dietary intervention may be a warranted mitigation strategy.

Introduction

Prenatal alcohol exposure (PAE) is the most common preventable cause of neurodevelopmental impairments in children (1). Maternal alcohol consumed during pregnancy passes through the placenta and acts as a teratogen in fetal tissues. PAE can cause Fetal Alcohol Spectrum Disorder (FASD), a diagnostic term encompassing the spectrum of physical, cognitive, behavioural and neurodevelopmental abnormalities that can occur in offspring as a result of PAE (2, 3). Although PAE is necessary to cause FASD, outcomes depend 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 gene regulation and development (4-6).

DNA methylation is an epigenetic mechanism of gene regulation involving the covalent modification of DNA cytosine bases, mostly within the cytosine-guanine sequence (CpG) of genomic DNA. This is facilitated by a family of methyltransferase enzymes that catalyse the addition of methyl groups to cytosines 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. Such modifications are mitotically stable throughout development. As DNA methylation is one of the control mechanisms for gene activity, alterations to this system by PAE could potentially disrupt fetal development and may be the cause of some of the difficulties experienced by people with FASD (7, 8). Previous studies on human participants (9-11) and animals (12-14) have implicated PAE as a mechanism of fetal DNA methylation disruption, either globally (11-13), and/or at specific gene regions (10, 13, 14) with limited replication of DNA methylation sites between studies (15). There has been much interest in the utility of DNA methylation as a potential biomarker for PAE, although results have been inconsistent (15). A mechanistic explanation for the effect of alcohol on DNA methylation is still lacking, but evidence from rodent (16) and human studies (17) suggests that 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. Indeed several clinical trials have explored the potential for dietary choline to attenuate the cognitive deficits caused by PAE in affected individuals (18, 19), or when administered during pregnancy (20, 21), with results suggesting a high methyl donor diet (HMD) could mitigate the adverse effects of PAE on various behavioural outcomes.

There are many limitations when studying PAE in humans such as a reliance on observational methodologies and self-reported alcohol consumption. In addition, it is difficult to measure and control for multiple factors that may influence fetal DNA methylation regulation, including diet and smoking. Thus, we employed an established experimental model of PAE combined with validation studies in human cohorts. We adopted the design of a murine study used by Kaminen-Ahola et al. (22) who performed site-specific DNA methylation sequencing on the Agouti allele in C57BL/6J mice exposed to moderate PAE

during the first eight days of pregnancy. This model is largely based on early PAE during the equivalent of the first trimester in humans when neurulation occurs and reflects a typical situation in which women may consume alcohol until pregnancy recognition (23). We employed whole genome bisulfite sequencing (WGBS) of fetal brain and liver to gain an unbiased view of the methylation state of all CpG dinucleotides while factoring the effect of tissue type on DNA methylation (24). Our WGBS approach also enabled sufficient sequencing coverage in the mouse genome to examine for alcohol-sensitive regions and the effects of a dietary intervention. We also assessed adult offspring mice for several behavioural outcomes that are known to be affected by PAE to determine if early, moderate PAE had any effects on these outcomes in adulthood, and if any of these effects were mitigated by the dietary intervention. The aims of this study were to: (1) determine if moderate PAE affected several behavioural outcomes in adult offspring mice; (2) identify candidate DNA methylation biomarkers for moderate PAE during the first trimester of pregnancy in a murine model; (3) investigate if HMD across pregnancy can mitigate any effects of PAE on offspring DNA methylation or behavioural outcomes; and (4) validate candidate biomarkers from the murine model within human cohorts for PAE or FASD.

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; H_20-HMD: -2.282 ± 0.918 g, P = 0.0177; PAE-HMD: -1.656 ± 0.814 g, P = 0.0493; figure 2a), 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 2b and S1c). There was no significant difference in the average litter size (6.525 ± 0.297 pups, figure 2c) and pup sex ratios (Figure 2d) 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 3).

Effects of PAE on DNA methylation in newborn mice tissue.

Whole-genome bisulfite sequencing (WGBS) of brain and liver samples from 16 newborn pups equally matched by treatment group and sex 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 evidence of differences in average methylation content (Figure S2).

To investigate region-specific effects of PAE on newborn DNA methylation, we conducted genome-wide testing on newborn NC mice. Comparison of methylation profiles between PAE and non-PAE groups within NC mice revealed 78 differentially methylated regions (DMRs) in the brain from 19,113,529 CpG sites tested after coverage filtering and 759 DMRs in the liver ($P < 0.05$ and delta > 0.05) from 19,335,801 CpG sites tested after coverage filtering (Figure 4a-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. Among these tissue agnostic regions was

the *Impact* gene on chromosome 18, which was hypomethylated in PAE+NC mice compared to H_20+NC mice in both the brain and liver (Figure 4c-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. The three genes associated with DMRs 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 4e-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 pathways, with neuron projection being the most significant (Figure 4g, 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 H_20+NC 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. The overall difference in DNA methylation with

PAE within brain and liver DMRs in the HMD mice were significantly lower than in the NC group mice (Figure 5a-d), with differences in DNA methylation with PAE more than 25% lower in some DMRs in the HMD mice compared to NC mice (Figure 5e-f).

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.

In the Australian AQUA cohort, we tested a total of 1,898 CpG sites that corresponded to mouse DMRs but did not find evidence of differential methylation (FDR < 0.05).

In the Lussier et al. cohort, differential testing of 2,048 CpG sites that were homologous to mouse DMRs identified statistical evidence of 47 DMR associations with FASD status (FDR P < 0.05). In models adjusted for participant age, sex, array number, and cell counts, 16/47 DMRs remained significant, seven of which had the same direction of effect observed in the murine model (Table 3). Visual comparison of methylation changes across these seven DMRs revealed striking differences in effect sizes between people with FASD and mice (Figure 6). We investigated the list of genes implicated by association with the 7 replicated DMRs using the GWAS and EWAS catalogue. Virtually all the genes of interest have been associated with haematological traits (*GADD45A, KLF1, PEX12, AP2B1, DNASE2*) and ageing phenotypes (*GADD45A, DNASE2, ZNF823, A2BP1*). GWAS studies have linked variations in these genes with traits related to facial morphology (*GADD45A* (46)), educational attainment (*AP2B1* (47), intelligence (*RP9* (48)), autism and schizophrenia (*ZNF823* (49)). The *Impact* gene, which contained a DMR in the brain and liver of newborn mice, did not contain any DMRs in any of the human cohorts (Figure 7).

Candidate Gene Analysis of previously defined alcohol sensitive regions.

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 we report on the effects in mice of maternal PAE on offspring DNA methylation, using a whole-genome bisulfite sequencing approach, which provides a comprehensive assessment of the mammalian DNA methylome. We found that moderate early (first trimester) PAE was associated with site-specific differences to DNA methylation in newborn pups but did not affect 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. Research evidence remains inconclusive about whether alcohol's effects on DNA methylation are stochastic, or deterministic, with varying reports of loss or gain of methylation in different tissues (15). We observed large differences in the effects of PAE on DNA methylation between brain and liver, only detecting two genomic regions and four genes that were similarly affected in both tissues from different germ layers. The latter finding suggests alcohol can perturb methylation events as early in pregnancy as during gastrulation, although on balance, our data were more supportive of a stochastic rather than a deterministic influence of alcohol on DNA methylation.

Although our murine model did not result in any significant differences in behavioural outcomes due to PAE, we identified site-specific differences in DNA methylation associated with early moderate PAE. Timing, dose, and other tissue-related factors all likely influence

the extent to which genome-regulation is perturbed and manifests as differences in DNA methylation. It is possible that more severe PAE might induce more consistent changes across tissues, whereas sustained exposure would conceivably induce tissue-specific methylation changes relating to the organ's metabolic processes.

We also found that DMRs were enriched in non-coding regions of the genome with regulatory potential suggesting alcohol may have broad effects on genome regulation. Despite this, the candidate gene analysis found reproducible associations in our model with previously 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 affects imprinting processes, although results from other studies are inconsistent (50, 51). We also replicated the alcohol-sensitive DMRs we found in the murine model in a human cohort of adolescents with FASD at genes with known associations with traits indicative of neurodevelopmental disorders (Table 3). That these effects were detectable in different tissues despite species-related differences is remarkable, and suggests that at least some methylation changes are stable. Collectively, our data 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 (52).

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 (53,

54), and mitigating effects on DNA methylation (55). 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.

This study provides a comprehensive assessment of the effects of PAE on DNA methylation using a well-established murine model of early moderate exposure. Our study design also utilized existing human data sets to explore the generalizability of the alcohol sensitive DMRs we found, which were replicated at seven human genes. Despite this, the level of PAE was insufficient to induce recognizable behavioural changes in adult littermates, and we did not assess markers of brain or liver physiology. We therefore had limited ability to infer pathogenic mechanisms from the methylation changes observed. We were also unable to determine the contribution of specific cell types within tissues to the methylation changes observed. In conclusion, we have identified novel targets with screening or diagnostic potential that warrant further study. Additionally, our findings add to the body of evidence that suggests a potential role for a dietary intervention to protect from the adverse effects of PAE.

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 epigenetic changes at the A^{vy} locus in Agouti mice (22) (Figure 1). This study received animal ethics approval from the Telethon Kids

Institute Animal Ethics Committee (Approval Number: 344). Sixty nulliparous C57BL/6JArc 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:

i. 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 moderate PAE during the first trimester of pregnancy in humans. Dams received non-acidified reverse osmosis water for the remainder of pregnancy and NC (Rat and Mouse Cubes, Speciality Feeds, Glen Forrest, Australia) throughout pregnancy.
ii. 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).
iii. H_20-NC (Water-Normal Chow): non-acidified water and NC throughout pregnancy.
iv. H_20-HMD (Water-High Methyl Donor diet): non-acidified water and HMD chow throughout pregnancy.

Whole-genome bisulfite sequencing of newborn mouse tissues

Two male and two female pups from each 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 -80ºC. Pups were selected from average sized litters for WGBS to minimize variability in PAE caused by differences in litter sizes. Remaining littermates grew until adulthood for behavioural testing. Ten milligrams of

tissue were extracted from each liver and brain sample. 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. 100ng 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 (Catalgoue 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:). WGBS was performed by Genomics WA sequencing core on a NovaSeq 6000 (Illumina) using 2x150bp 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 9.69x (range: 6.51-12.12x). Raw fastq files were mapped to the mm10 mouse reference genome with BSseeker 2 (version 2.1.8) (25) and CG-maptools (version number 0.1.2) (26) using a custom bioinformatics pipeline.

Behavioural testing in adult mice

Littermates who were not sacrificed were reared on normal chow and drinking water *ad libitum* until adulthood (~8 weeks after birth) when they underwent behavioural testing for five neurodevelopmental functions including locomotor activity, anxiety, spatial recognition, memory, motor coordination and balance. Behavioural tests included the open field test

(locomotor activity, anxiety) (27), object recognition test (locomotor activity, spatial recognition) (28), object in place test (locomotor activity, spatial recognition) (29), elevated plus maze test (locomotor activity, anxiety) (30), and rotarod test (motor coordination, balance) (31).

Validation studies in human cohorts

Validation studies were undertaken in human cohorts with existing genome-wide DNA methylation data sets and matching PAE data. These are described below:

1. The Asking Questions About Alcohol in Pregnancy (AQUA) cohort (32) is an observational Australian cohort of 1,570 mother-child pairs. Timing, dose, and frequency of alcohol consumption data were collected once in each trimester using detailed questionnaires that were designed with consumer input to encourage honest and accurate reporting (33). Genome-wide DNA methylation measurements were available for 147 newborns from buccal swabs assayed on the IlluminaHumanMethylation450k array. Newborns were classified according to the level of maternal alcohol use during pregnancy as abstainers (51 subjects), moderate discontinued (40 subjects), moderate sustained (49 subjects), or high sustained (7 subjects) (34).
2. The Lussier et al. cohort (9) is a cohort study for clinical diagnosis of FASD in Canadian children. The cohort (GSE80261) initially contained 216 children (112 FASD and 104 controls) from the NeuroDevNet FASD cohort. Genome-wide DNA methylation measures were taken from buccal swabs assayed on the IlluminaHumanMethylation450k array. We performed initial quality filtering of the data and included only Caucasians to avoid ethnic confounding (ethnicity chi-squared

$P = 8.28*10^{-14}$ before filtering). This reduced the sample size to 118 individuals (30 FASD and 88 controls).

3. The Cobben et al. cohort (35) is a cohort study for clinical diagnosis of FASD in Canadian children. We assessed the combined discovery (GSE112987) and replication (GSE113012) cohorts, which contained a total of 138 children (92 FASD and 64 controls). Genome-wide DNA methylation measures were taken from blood samples assayed on the IlluminaHumanMethylation450k array.

For the AQUA and Cobben et al. datasets, raw .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. The pre-processed Lussier et al. cohort dataset was obtained directly from the Gene Expression Omnibus database using the *getGenomicRatioSetFromGEO* command from *minfi*.

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.

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. The exceptions were the 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.

We converted CGmap files from each mouse sample into CG methylation bsseq objects, and then combined the bsseq objects from each sample. We filtered the data to include only the autosomes and then combined reads from mice in the same treatment group using the *collapseBSseq* function, to maximise coverage prior to differential methylation analysis. The X chromosome was excluded as we were searching for a PAE biomarker that was independent of sex. CpG sites with an aggregated coverage below 10x 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 tissues using a Bayesian hierarchical model comparing exposed and unexposed groups using the Wald test with smoothing, implemented in the R package *DSS* (39). 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. For validation studies in humans, differential testing of mouse DMRs was carried out using the R package *DMRcate* (40) for each dataset and DMRs were declared as minimum smoothed false-discovery rate (FDR) < 0.05. Gene ontology analysis was carried out using Gene Set Enrichment Analysis (41), which assessed brain and liver DMRs for transcription start sites and associated biological processes.

Brain and liver DMRs were tested for enrichment within ENCODE Project data sets (42) 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, which identified 37 candidate genes ((15), 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 NC mice using the *callDML* feature in DSS.

Competing Interest Statement

The authors declare no competing interests.

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Author Contributions

DM, MS, AL, RL, DH, EM, JC, JH, JF, SB, MB, EE contributed to the study design and funding application. EC, AL performed the mouse work including administering the mating, treatments, measurements, monitoring and extracting biological samples. EC, AL, MB performed the mouse behavioural testing. CB analysed the videos from the mouse behavioural testing. MB prepared the whole-genome bisulfite sequencing libraries and

performed the data analyses. NK assisted with preparing whole-genome bisulfite sequencing libraries. SB, DM provided advice and support on the data analysis. JH, EM contributed human datasets for reproducibility analysis. MB, DM drafted the manuscript. MS, AL, RL, DH, EM, JC, JH, JF, SB, EE, NK contributed to the development and editing of the manuscript.

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Table 1: Table of DMRs identified in the intronic regions of genes that contained DMRs in both the brain and liver. Δmeth indicates the percentage change in average methylation level within the DMR with PAE compared to non-PAE mice.

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

Table 2: Number and percentage of brain and liver DMRs that overlap with tissue-specific atacseq, H3K4me1 and H3K27ac regions obtained at 0 days postnatal from the ENCODE database. P-values for permutation testing using a randomisation strategy.

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 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.

DMR	**organism**	**tissue**	**chr**	**start**	**end**	**width**	**no. cpgs**	**FDR**	**meandiff**	**gene**
1	human	buccal	1	68151571	68152310	740	5	0.028636	-0.00497	*GADD45A*
2	human	buccal	19	13000782	13002357	1576	11	0.000197	-0.00203	*GCDH*
3	human	buccal	7	33148815	33149316	502	11	0.001149	-0.00011	*RP9*
4	human	buccal	17	33905444	33905888	445	14	0.000171	-0.00359	*AP2B1, PEX12*
5	human	buccal	17	27181503	27182342	840	11	0.018536	-0.00246	*ERAL1, FAM222B*
6	human	buccal	19	12992181	12992479	299	9	0.037431	-0.00179	*CTD-2265O21.7, DNASE2*
7	human	buccal	19	11849531	11850013	483	9	0.022724	-0.00244	*ZNF823*
1	mouse	liver	6	67034885	67035082	197	4	<0.05	-0.220833	*E230016M11Rik*
2	mouse	liver	8	84901298	84901544	246	5	<0.05	-0.234457	*Klf1*
3	mouse	liver	9	22453836	22453893	57	5	<0.05	-0.226427	*Rp9*
4	mouse	brain	14	21403570	21403622	52	4	<0.05	-0.234193	*Adk*
5	mouse	liver	11	78069463	78070002	539	9	<0.05	-0.255864	*Mir144, Mir451a*
6	mouse	liver	11	78072079	78072313	234	4	<0.05	-0.215227	*Mir144, Mir451a*
7	mouse	liver	2	177091927	177092945	1018	5	<0.05	-0.224354	Intergenic

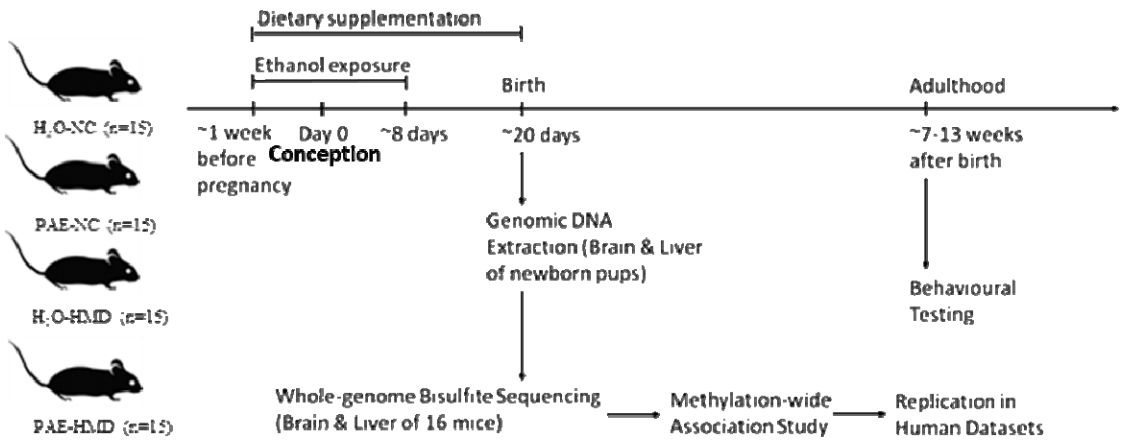

Figure 1. Overview of PAE model.

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 (H_20). The PAE and H_20 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.

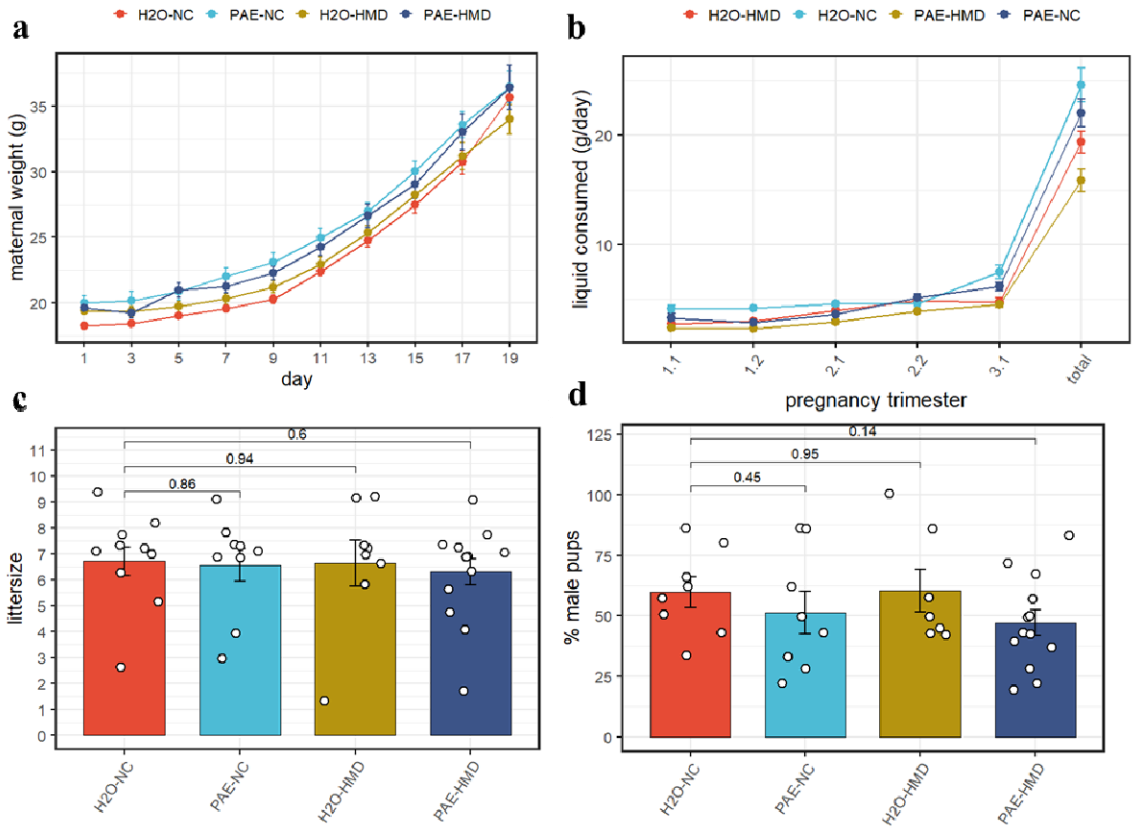

Figure 2. PAE and HMD had varied effects on dam characteristics.

(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 raw means and SEM. NC = normal chow, HMD = high methyl diet, PAE = prenatal alcohol exposure. Comparisons show p-value by unpaired t-test compared to the H_20-NC baseline treatment group.

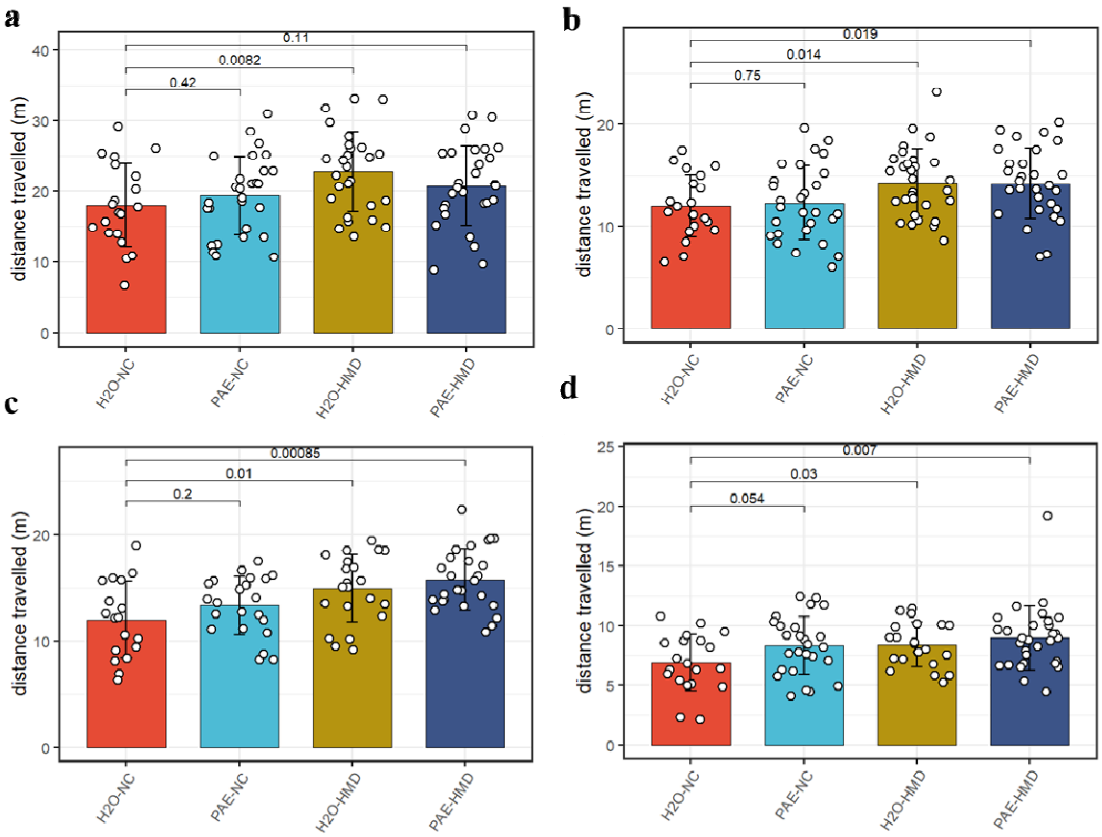

Figure 3. HMD was associated with increased locomotor activity.

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.

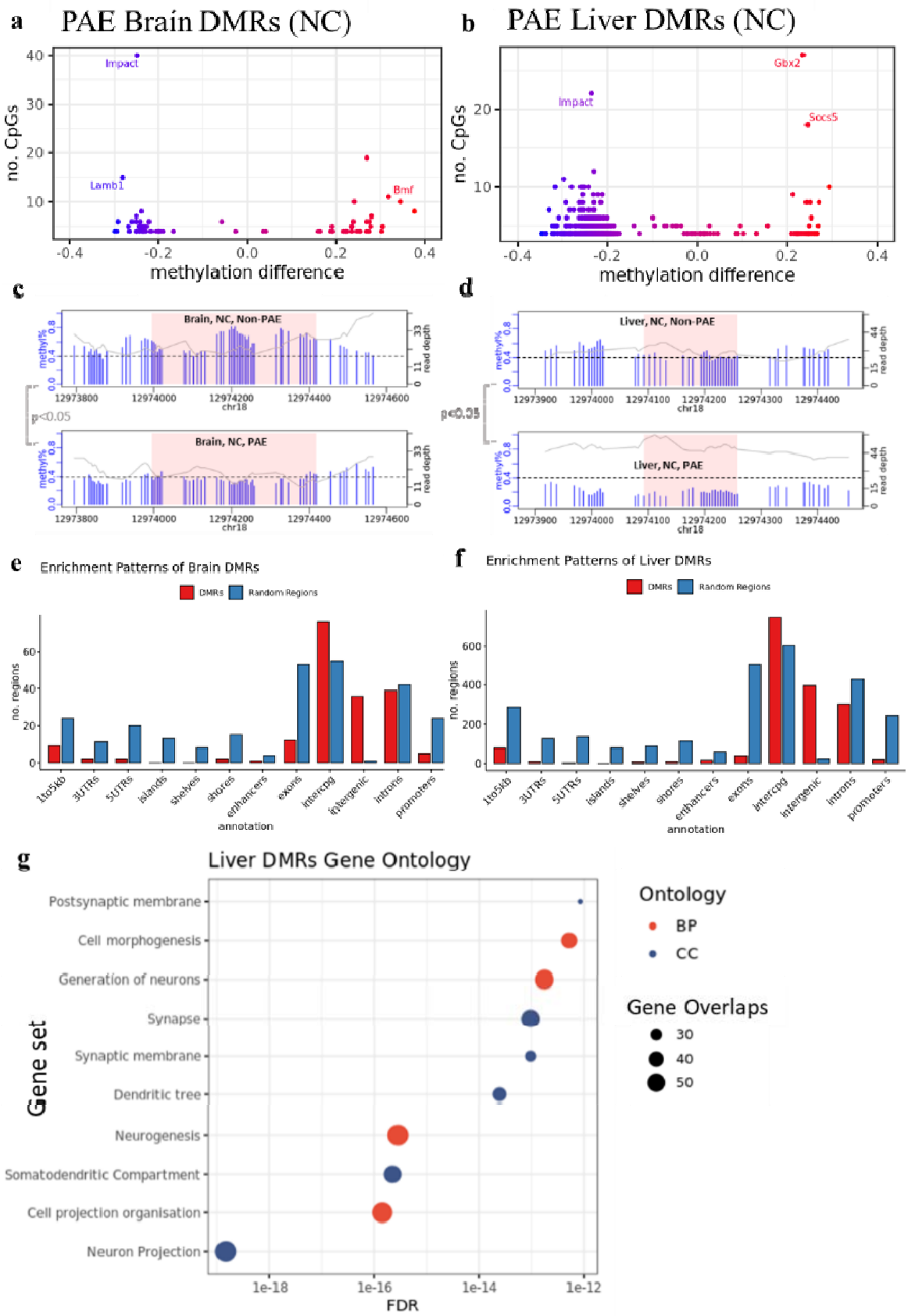
a
PAE Brain DMRs (NC)
no. CpGs
methylation difference
Impact
Lamb1
Bmf
b
PAE Liver DMRs (NC)
Gbx2
Socs5
c
Brain, NC, Non-PAE
Brain, NC, PAE
p<0.05
d
Liver, NC, Non-PAE
Liver, NC, PAE
e
Enrichment Patterns of Brain DMRs
DMRs
Random Regions
no. regions
annotation
f
Enrichment Patterns of Liver DMRs
g
Liver DMRs Gene Ontology
Gene set
Postsynaptic membrane
Cell morphogenesis
Generation of neurons
Synapse
Synaptic membrane
Dendritic tree
Neurogenesis
Somatodendritic Compartment
Cell projection organisation
Neuron Projection
FDR
Ontology
BP
CC
Gene Overlaps

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Figure 4. 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.

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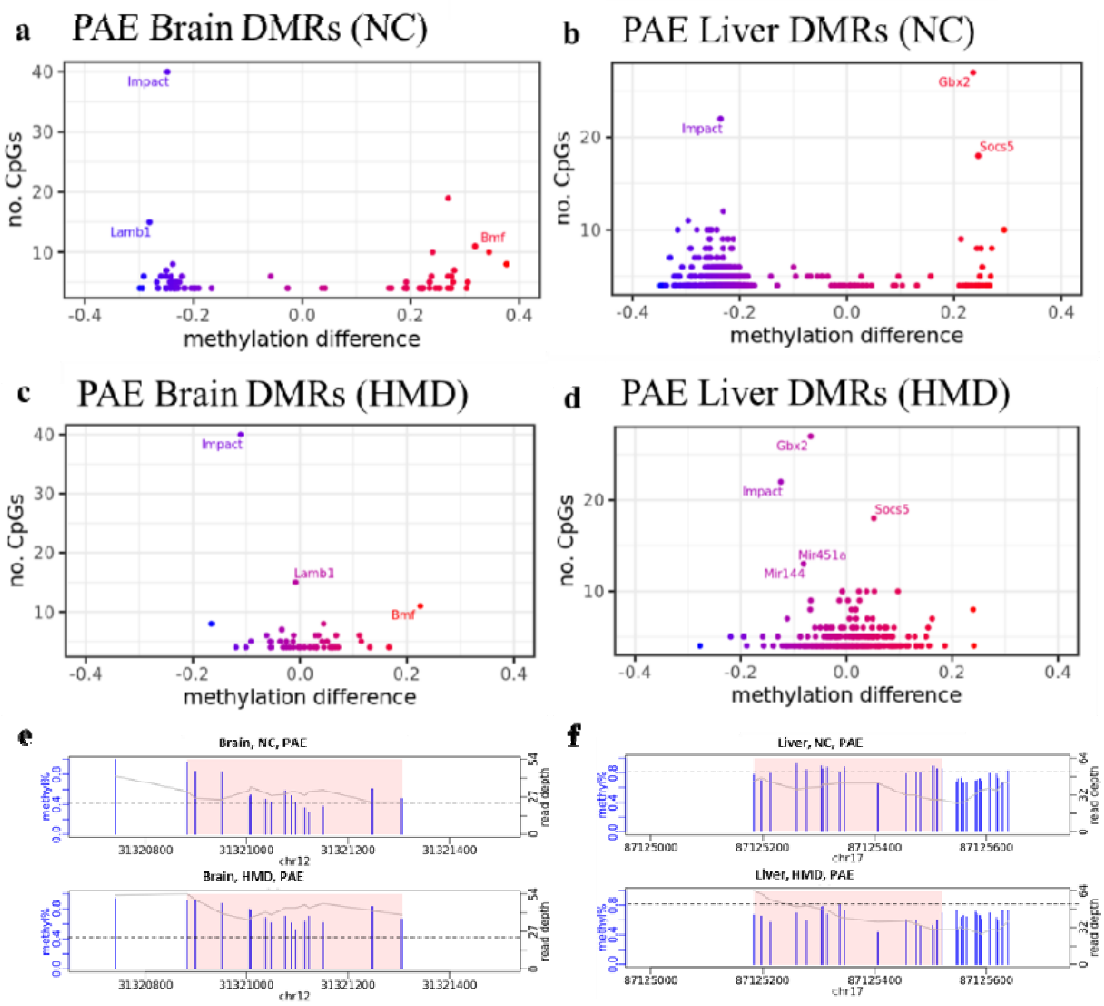

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Figure 5. HMD partially mitigated effects of PAE on offspring DNA methylation.

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.

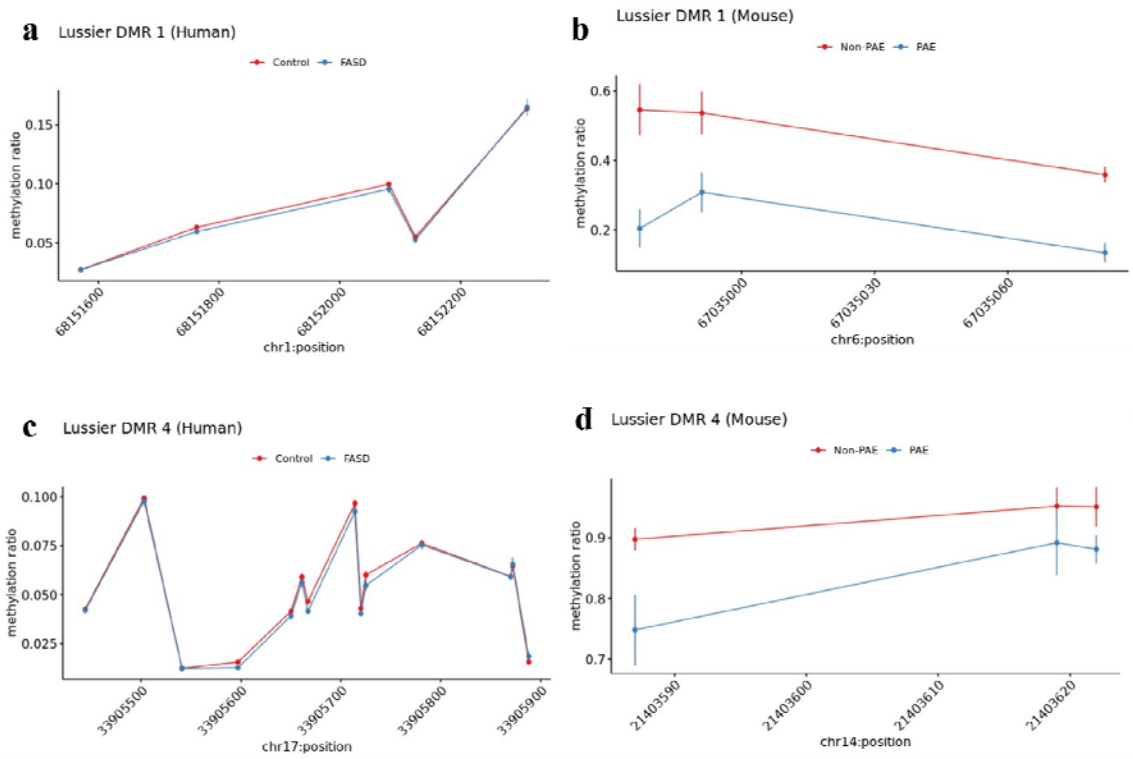

Figure 6. Seven PAE DMRs identified in the murine model were successfully replicated in the Lussier et al. human FASD cohort.

Examples of two PAE DMRs that were significantly hypomethylated with a clinical diagnosis of FASD in the Lussier et al. cohort (a and c), while their mouse liftover DMR was also significantly hypomethylated with PAE in the murine model experiment (b and d).

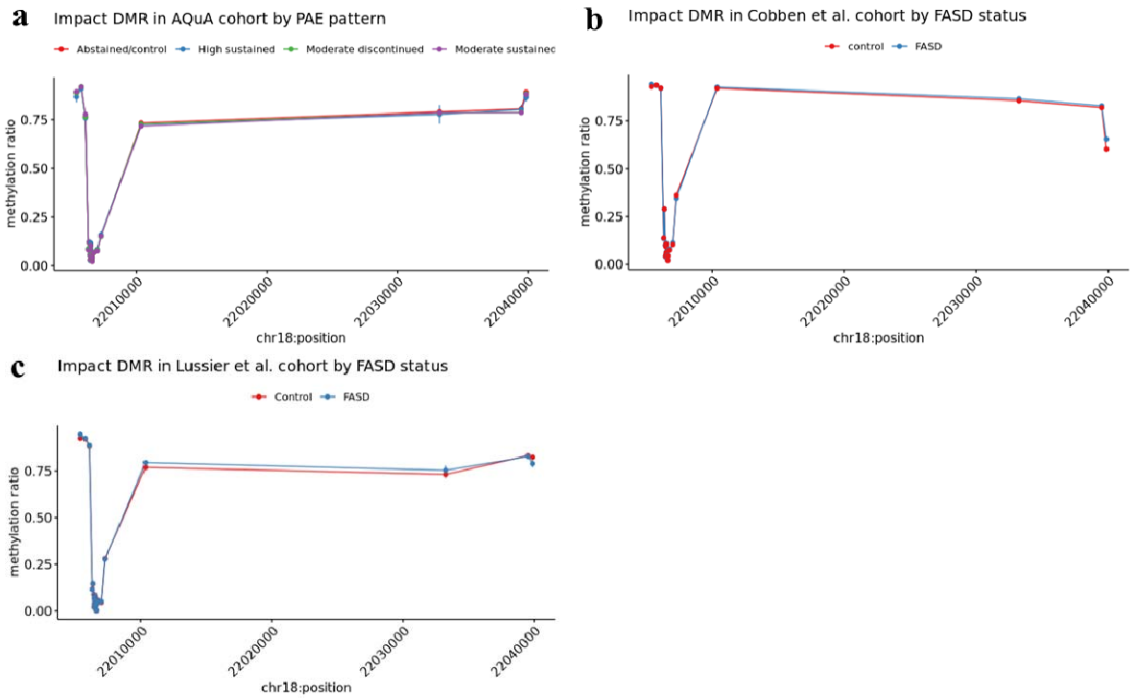

Figure 7. Methylation in the *Impact* gene and surrounding regions was not significantly affected by PAE or FASD in human cohorts.

The *Impact* gene, which contained DMRs significantly associated with PAE in the mouse brain and liver, was not significantly associated with (a) PAE in the AQuA cohort, or a clinical diagnosis of FASD in the (b) Cobben et al. cohort, or (c) Lussier et al. cohort.