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RESEARCH ARTICLE

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# Mitigating the detrimental developmental impact of early fetal alcohol exposure using a maternal methyl donor-enriched diet

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## Abstract

Fetal alcohol exposure at any stage of pregnancy can lead to fetal alcohol spectrum disorder (FASD), a group of life-long conditions characterized by congenital malformations, as well as cognitive, behavioral, and emotional impairments. The teratogenic effects of alcohol have long been publicized; yet fetal alcohol exposure is one of the most common preventable causes of birth defects. Currently, alcohol abstinence during pregnancy is the best and only way to prevent FASD. However, alcohol consumption remains astoundingly prevalent among pregnant women; therefore, additional measures need to be made available to help protect the developing embryo before irreparable damage is done. Maternal nutritional interventions using methyl donors have been investigated as potential preventative measures to mitigate the adverse effects of fetal alcohol exposure. Here, we show that a single acute preimplantation (E2.5; 8-cell stage) fetal alcohol exposure (2×2.5 g/kg ethanol with a 2h interval) in mice leads to long-term FASD-like morphological phenotypes (e.g. growth restriction, brain malformations, skeletal delays) in late-gestation embryos (E18.5) and demonstrate that supplementing the maternal diet with a combination of four methyl donor nutrients, folic acid, choline, betaine, and vitamin B12, prior to conception and throughout gestation effectively reduces the incidence and severity of alcohol-induced morphological defects without altering DNA methylation status of imprinting control regions and regulation of associated imprinted genes. This study clearly supports that preimplantation embryos are vulnerable to the teratogenic effects of alcohol, emphasizes the dangers of maternal alcohol consumption during early gestation, and provides a potential proactive maternal nutritional intervention to minimize FASD progression, reinforcing the importance of adequate preconception and prenatal nutrition.

**Abbreviations:** CpG, cytosine-phosphate-guanine dinucleotide; Ctl, control; DOHaD, developmental origins of health and disease; E, embryonic day; ENR, methyl donor-enriched maternal diet; EtOH, ethanol; FASD, fetal alcohol spectrum disorder; H19, H19 imprinted maternally expressed transcript; ICR, imprinting control region; IGF2, insulin-like growth factor 2; IGF2R, insulin-like growth factor 2 receptor; LTR, long terminal repeats; SAM, S-adenosylmethionine; STD, standard maternal diet.

Mélanie Breton-Larrivée and Elizabeth Elder contributed equally.

**KEYWORDS**
DNA methylation, DOHaD, embryonic development, environmental exposure, FASD, fetal alcohol exposure, maternal nutrition, methyl donors

## 1 | INTRODUCTION

Fetal alcohol spectrum disorder (FASD) includes a broad range of adverse developmental conditions that result from maternal alcohol intake during any stage of pregnancy.[1–5] Even though alcohol is a well-known teratogen and there are many awareness-raising initiatives for healthcare providers and the general public,[6–8] an estimated 10% of pregnant women consume alcohol globally, of which over one quarter are reported to engage in binge drinking (single acute episode) specifically.[9–11] The global prevalence of FASD among children and youth is estimated at 0.8%, affecting approximately 0.9% in the Americas, 2% in European countries, and 11% in South Africa.[12] Individuals with FASD can exhibit a variety of mild to severe phenotypes including congenital anomalies (e.g. craniofacial malformations, ossification delays, intrauterine growth retardation, structural brain abnormalities), as well as cognitive (e.g. intellectual and learning disabilities), behavioral, and emotional disorders.[13–16] Various factors such as the amount of alcohol consumed and the timing, duration, and frequency of the exposure during embryonic development may contribute to the phenotypic variability of FASD.[3,17,18] For instance, studies suggest that a single acute (i.e., binge) exposure (peak blood alcohol level of ≥200 mg/dL) can be more damaging to the developing embryo than a chronic, milder exposure (blood alcohol level of ≤100 mg/dL).[19–22] Moreover, different inbred mouse strains have been shown to exhibit variable responses to fetal alcohol exposure,[23–26] suggesting that genetic differences play a part in interindividual susceptibility and resistance to the teratogenic effects of alcohol. Secondary factors, such as poor nutritional status of the mother[27,28] and exposure to early life stress,[29] have also been shown to contribute to the severity of FASD phenotypes.

Fetal alcohol spectrum disorder animal models have shown that alcohol exposure interferes with DNA methylation pathways in the developing embryo[30–35] and DNA methylation alterations in blood and buccal cells have been associated with FASD in cohorts of children.[36–38] The regulation of gene expression by DNA methylation mechanisms rely on one-carbon metabolism, composed of the interdependent folate and methionine cycles, through which S-adenosylmethionine (SAM) is produced, the universal methyl donor in cellular methylation reactions.[39–41] Folate, choline, betaine, and vitamin B12 are essential nutrients involved in SAM synthesis, and collectively referred to as methyl donors. Alcohol use can reduce bioavailability of many nutrients including folate and vitamin B12[42–46] as well as dysregulate one-carbon metabolism pathways[47–50] and DNA methylation mechanisms.[51–54] During pregnancy, women are vulnerable to deficiencies in methyl donor nutrients as maternal metabolic demand for these nutrients increases to support fetal and placental development.[55–57] Consequently, many women do not consume the recommended dietary intake for several of these methyl donor nutrients during pregnancy.[58–61] Severe congenital abnormalities have been associated with maternal nutritional deficiencies in methyl donors. For example, maternal folate deficiency can lead to neural tube defects,[62,63] and maternal choline deficiency in mice has been shown to hinder development of the cerebral cortex[64] and hippocampus[65] and alter cognitive function in offspring.[66] Furthermore, studies have demonstrated that the teratogenic effects of alcohol in rats and mice can be intensified by suboptimal intake of methyl donors during pregnancy, worsening developmental outcomes in offspring.[67,68]

Although we know that FASD can stem from fetal alcohol exposure taking place at any time during pregnancy, preimplantation, which occurs between fertilization and embryonic day 4.5 (E4.5) in mice or E7-9 in humans,[69–71] is arguably the stage of development most at-risk for unintended exposure. The likelihood of accidental fetal alcohol exposure during preimplantation is substantial considering that human chorionic gonadotropin (i.e., the hormone detected by pregnancy tests) is only produced after implantation of the embryo,[72] that 40% of pregnancies are unplanned,[73] and that there is an increasing rate of alcohol consumption and binge drinking behavior among women of reproductive age,[74–77] possibly even more so since the COVID-19 pandemic.[78] During preimplantation, the embryo undergoes reprogramming of epigenetic modifications, notably DNA methylation, which is crucial for normal gene expression regulation during development[79–82] and is particularly vulnerable to unfavorable in utero environments,[83] for example, assisted reproductive technologies,[84–88] nutritional imbalances,[89–91] or exposures to chemical compounds (e.g. insecticides)[92] and alcohol.[30,33,35] Defective embryonic epigenetic reprogramming can result in DNA methylation dysregulation at imprinting control regions (ICRs),[93–97] which can lead to a myriad of developmental disorders[98–101] as ICRs control a cohort of genes (i.e., imprinted genes) that regulate growth and development of the fetus and placenta,[102]

for example, *H19* (H19 imprinted maternally expressed transcript),[103] *IGF2* (insulin-like growth factor 2),[104] and *IGF2R* (insulin-like growth factor 2 receptor).[105] FASD in vivo models have shown that preimplantation alcohol exposure can induce permanent FASD-like physical, behavioral, and molecular (i.e., epigenetic, transcriptomic) phenotypes observable throughout later stages of development.[30,33,106–109] In a previous study, we showed that a single acute preimplantation (E2.5) alcohol exposure in mice led to severe morphological defects (e.g. growth restriction, brain malformations) and DNA methylation dysregulation at some ICRs and neurodevelopmental loci in forebrain at midgestation (E10.5).[30] However, because of the all-or-nothing tenet, meaning the embryo would either die prior to implantation or survive unharmed, research has been mainly focused on the effects of alcohol exposure during postimplantation developmental stages.[20,23–26,34,110–113] Further research on the specific and long-term teratogenic effects of alcohol exposure during preimplantation is necessary to have a more comprehensive understanding of FASD etiology.

Despite FASD being a serious widespread concern with lifelong health consequences, there are no proactive therapeutic measures in place to mitigate the effects of fetal alcohol exposure before birth. Studies in animal models and human subjects suggest that maternal nutritional supplementation of various methyl donor nutrients may have a protective effect, significantly minimizing the incidence of developmental defects and behavioral impairments as well as rescuing DNA methylation alterations in fetuses and offspring exposed to alcohol in utero.[110–118] However, in most of these studies, only one or two types of methyl donor nutrients were supplemented in the maternal diet, which could lead to unbalanced metabolism cycles,[119] and the amounts supplemented were strikingly higher than standard rodent chow (e.g. NIH-31 rodent formula) levels (i.e., 7.5- to 30-fold higher). Excess supplementation of various methyl donor nutrients in the maternal diet has been linked to DNA methylation alterations in cow and mice offspring[120,121] and high maternal intake of folic acid has been associated with insulin resistance in children[122] and rat offspring[123] and to altered synaptic transmission and increased susceptibility to seizures in rat offspring.[124] Since high-dose supplementation can be harmful for the developing fetus and increase the risk of disease, we investigated if moderate supplementation of a combination of the four methyl donor nutrients folic acid, choline, betaine, and vitamin B12 in the maternal diet prior to and throughout pregnancy could mitigate late-gestation (E18.5) detrimental effects (i.e., morphological defects) resulting from a single acute fetal alcohol exposure during mouse preimplantation (E2.5), without enhancing the risk of DNA methylation dysregulation at imprinting control regions.

## 2 | MATERIALS AND METHODS

### 2.1 | Maternal diets

Animal work was approved by the *Comité institutionnel de bonnes pratiques animales en recherche* of the Centre Hospitalier Universitaire Sainte-Justine Research Center under the guidance of the Canadian Council on Animal Care. For all experiments, mice were maintained on a 12-h light/dark cycle. At 4 weeks of age, female C57BL/6 mice ($n$ = 8–9/condition) (Charles River, Canada) were fed ad libitum one of two defined diets for 4 weeks prior to mating, to allow stabilization of nutrients in serum,[125] and were fed the same diets throughout gestation until embryo collection (Figure 1). The NIH-31 rodent formula (Envigo) containing 1.89 g/kg choline, 2 mg/kg folic acid and 0.06 mg/kg B12 was used as the standard diet, and a modified formula supplemented with methyl donors (5.76 g/kg choline, 5 g/kg betaine, 5 mg/kg folic acid, 0.5 mg/kg vitamin B12) as the enriched diet. After 4 weeks on their respective diet, females were mated with C57BL/6 males of same age (Charles River, Canada) that were kept on the standard diet. Females that showed copulatory plugs the next morning were considered pregnant with day 0.5 embryos (E0.5). These females were separated from the males and housed together with unlimited access to food (standard or enriched diet) and water.

### 2.2 | Acute preimplantation alcohol exposure

Using a recognized preimplantation acute alcohol exposure paradigm,[30] the pregnant mice were subcutaneously injected at E2.5 (8-cell embryos) with two doses of 2.5 g/kg ethanol (EtOH-exposed), or 0.15 M saline (control) with a 2-h interval (Figure 1). Pregnant females under this paradigm reach a peak blood alcohol concentration (BAC) of 284.27 mg/dL (3 h post-exposure) with an average of 158.31 mg/dL over a 4-h window.[30] Females with the same diet regime or exposure treatment were housed together with negligible handling during gestation until embryo collection at E18.5 (late gestation).

### 2.3 | Morphological analysis

At E18.5, embryos and their placenta were collected for morphological analysis. Placentae were weighed and then

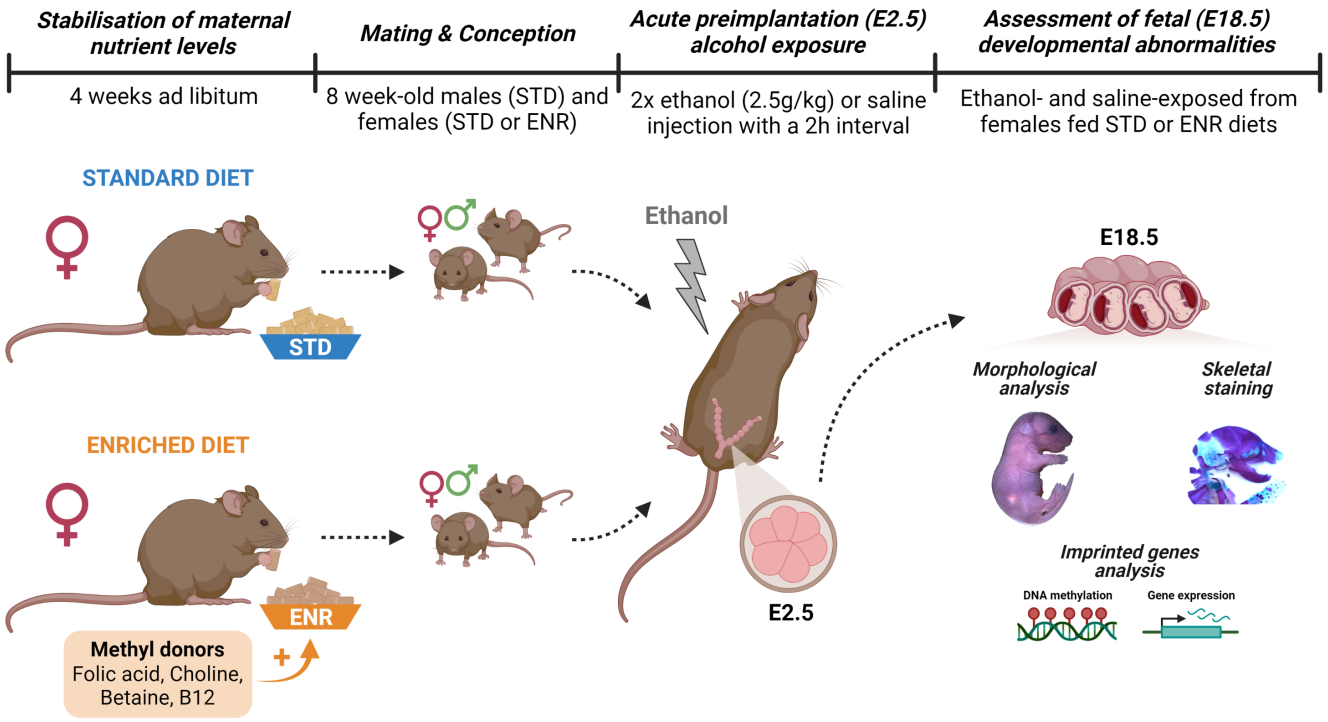



**FIGURE 1** Mouse model combining a maternal methyl donor-enriched diet and a single acute preimplantation alcohol exposure. CB57BL/6 females were fed a standard or a methyl donor-enriched (folic acid, choline, betaine, and vitamin B12) diet for 4 weeks to stabilize serum nutrient levels prior to mating with CB57BL/6 males kept on a standard diet; Respective maternal diets were continued throughout gestation. Pregnant females were subjected to a single acute alcohol exposure (2.5 g/kg EtOH) or 0.15 M saline solution (control) by two subcutaneous injections of equivalent volumes with a 2-h interval to target E2.5 stage embryos (~8-cell stage). E18.5 embryos were harvested for morphological analysis, skeletal staining, and analysis of DNA methylation and gene expression of imprinted genes in forebrain tissue. Created with BioRender.com.

preserved at −80°C. Using a Leica stereo microscope and the Application Suite X (LAS X) software, embryo morphometric measurements were performed for; crown-rump length (top of the head to the base of the tail), head length (occipital part of the head to the nasal process), head height (top of the head to the beginning of the torso), head width (width of the top of the head perpendicular to the ears), and distance between the eyes (space between the inner canthi). Morphological defects (e.g. growth restriction, eye anomaly, or any other abnormal feature) were evaluated. Next, whole E18.5 embryos were weighed, embryonic brains were extracted, weighed, and assessed for brain malformations, and forebrains were isolated for molecular analysis. Samples were kept at −80°C for further experiments. The sex of each embryo was determined by amplifying *Kdm5c* (X chromosome) and *Kdm5d* (Y chromosome) by qPCR (forward primer: CTGAAGCTTTT GGCTTTGAG, reverse primer: CCACTGCCAAATTC TTTGG) on yolk sac DNA extracted according to QIAamp DNA Mini Kit protocol (Qiagen). Number and sex of embryos in each condition group: STD-Ctl (male: 39, female: 29, total: 68), ENR-Ctl (male: 36, female: 33, total: 69), STD-EtOH (male: 41, female: 29, total: 70), ENR-EtOH (male: 44, female: 31, total: 75).

## 2.4 | Skeletal preparation

The alcian blue and alizarin red staining protocol was adapted from Lambrot et al.[126] Embryos at E18.5 were fixed in 80% ethanol for 24 h. The skin and viscera were removed using forceps, and the fixing solution was replaced with 95% EtOH for 24 h. The embryo cartilages were stained for 24 h in alcian blue solution and washed in 95% EtOH for 5 h. To remove muscles, embryos were treated with 2% KOH solution for 24 h. The bones were then stained in 1% KOH/0.012% alizarin red S solution overnight. They were washed in 1% KOH/20% glycerol to clear the skeletons and then stored in 50% glycerol/50% EtOH. Imaging was done with a Leica stereo microscope in bright field. Number of embryos in each condition group: STD-Ctl-normal morphology ($n = 6$), ENR-Ctl-normal morphology ($n = 6$), STD-EtOH-normal morphology ($n = 11$), ENR-EtOH-normal morphology ($n = 10$),

STD-EtOH-growth restricted ($n = 7$), ENR-EtOH-growth restricted ($n = 6$).

## 2.5 | DNA extraction and DNA methylation analysis

Frozen E18.5 forebrains were first ground into powder using liquid nitrogen with a mortar and pestle and DNA was extracted according to QIAamp DNA Mini Kit protocol (Qiagen). Genomic DNA (1 µg) was then bisulfite-converted using the EZ DNA methylation Gold kit (Zymo Research) according to the manufacturer's protocol and eluted in 100 µL EB buffer. The samples were then diluted to obtain a concentration of 1 ng/µL. Number of embryos in each condition group: STD-Ctl-normal morphology ($n = 6$), ENR-Ctl-normal morphology ($n = 6$), STD-EtOH-normal morphology ($n = 6$), ENR-EtOH-normal morphology ($n = 6$), STD-EtOH-growth restricted ($n = 4$), ENR-EtOH-growth restricted ($n = 4$).

DNA methylation levels of imprinted control regions (ICR) in forebrain samples were quantified using the *Implicon* technique adapted from Klobučar et al.[127] First, we designed ICR-specific bisulfite sequencing primers and added the sequence "CTACACGACGCTCTTCCGATCT" to the forward primer and the sequence "TGCTGAACCGCTCTTCCGATCTNNNNNNNN" to the reverse primer (N corresponds to a random nucleotide) (Table S9). The random nucleotide sequences serve as unique molecular identifiers (UMI) for downstream bioinformatics analysis. For the first *Implicon* PCR reaction (PCR1), we used 1 ng of bisulfite-converted DNA, 0.375 µM of pooled primers (forward and reverse), and KAPA Hifi Uracil+Ready Mix in a final volume of 8 µL for each sample and ICR. For each sample, the different amplified ICRs were pooled, cleaned up with 1.5× AMPure XP beads, and eluted in 20 µL EB buffer. This PCR pool was quantified by Qubit and diluted to a concentration of 0.40 ng/µL in a volume of 20 µL EB buffer. In the second PCR reaction, primers containing the barcoded Illumina adapters were used which ensures that each sample is amplified with a unique reverse barcoded adapter. The diluted PCR1 pool (20 µL) was amplified with 0.2 µM of Illumina PE1.0 primer, 0.2 µM of Illumina custom PE2.0 primer, and KAPA Hifi Uracil+Ready Mix in a final volume of 50 µL. After amplification, PCR2 reaction cleanup was done using 1X AMPure XP beads and eluted in 20 µL EB buffer. The libraries were sequenced with the Illumina Miseq platform to generate 250 bp paired-end reads (62 500 reads minimum per library).

## 2.6 | Bioinformatics analysis of DNA methylation

Quantification analysis of ICR DNA methylation levels was based on the *Implicon* bioinformatics pipeline.[127] First, the eight-random nucleotide sequences serving as UMIs were filtered out and inserted into the readID of the two sequencing reads for deduplication afterward. Next, the lower quality sequences and the adaptor sequences were filtered out of the raw sequencing reads. These two steps were accomplished with Trim Galore (version 0.6.6). The sequencing reads were aligned to the mouse reference genome mm10 using Bismark (version 0.22) which is a specific program for mapping bisulfite-treated sequencing reads and performing DNA methylation calls. After alignment, the reads were deduplicated and CpGs were extracted with Bismark. Only CpGs with a minimum sequencing coverage of 10× were retained and the average methylation level was calculated according to the coverage. To compare average CpG methylation levels between samples, we retained only the sequenced CpGs that were common among all samples.

## 2.7 | Gene expression analysis

Gene expression levels of imprinted genes and *Hprt1* (endogenous control) in E18.5 forebrains (frozen powder) of same embryos used for DNA methylation analysis were measured by quantitative PCR (qPCR) using total RNA extracted with the RNeasy Mini kit (Qiagen). Briefly, 1.5 µg of RNA for each sample was converted to cDNA using the SuperScript IV Reverse Transcriptase (Thermo Fisher). Primers specific to the regions of interest were designed with Primer3 (version 0.4.0) (Table S9) to obtain amplicons ranging from 60 to 150 bp. qPCR was performed using the SensiFAST™ SYBR® No-ROX Kit on a LightCycler 96 (Roche Life Science). Expression levels were calculated with the $2^{-\Delta Cq}$ method[128] using the *Hprt1* gene for normalization. Mean normalized expression of the STD-Ctl group was used to calculate relative expression. Number of embryos in each condition group: STD-Ctl-normal morphology ($n = 6$), ENR-Ctl-normal morphology ($n = 6$), STD-EtOH-normal morphology ($n = 6$), ENR-EtOH-normal morphology ($n = 6$), STD-EtOH-growth restricted ($n = 4$), ENR-EtOH-growth restricted ($n = 4$).

## 2.8 | Statistical analysis

Statistical analysis of weight and weight gain of dams prior to mating was done using the Shapiro–Wilk normality test

and Levene's test for homogeneity of variance followed by the two-sample *t* test to compare weight means or the Mann–Whitney–Wilcoxon test for weight gain means. Statistical analysis of the frequencies of total morphological defects or total ossification delays in embryos was done using pairwise chi-squared tests with Yates continuity correction. For all other assays, statistical analysis was done as follows. First, the Shapiro–Wilk test was used to verify normality. Then, to compare the different groups of conditions, parametric tests were used if all groups of conditions passed the normality test and non-parametric tests were used if at least one condition group failed the normality test. Parametric tests: Levene's test was used to verify homogeneity of variance among the groups of conditions and then one-way ANOVA with pairwise comparisons and, if required, Welch's correction for unequal variances was performed with Benjamini–Hochberg *p*-value adjustment. Non-parametric tests: Kruskal–Wallis test and Pairwise Wilcoxon Rank Sum tests were performed with Benjamini–Hochberg *p*-value adjustment.

## 3 | RESULTS

### 3.1 | Maternal diet enriched in methyl donors reduces morphological abnormalities associated with early fetal alcohol exposure

To avoid the increased risk of harmful outcomes associated with excessive methyl donor supplementation, we investigated if a moderate increase in nutrient availability of folic acid, choline, betaine, and vitamin B12 in the maternal diet could counteract the detrimental effects of early fetal alcohol exposure. Dams were fed either a standard (STD) or a methyl donor-enriched diet (ENR) 4 weeks prior to mating[125] with males fed the standard diet (Figure 1). Females fed the standard or enriched diet showed no difference in weight gain curves (Figure S1, Table S1). Then, using our established preclinical mouse model of acute preimplantation (E2.5) alcohol exposure,[30] females on standard or enriched diets were subjected to a single acute level of ethanol (EtOH) or saline control solution and were kept on their respective diet until embryo collection at late gestation (E18.5) (Figure 1). The average number of embryos per litter was not significantly affected by either the ethanol exposure or the enriched diet (Figure S2, Table S5). Assessment of embryonic morphometric measurements ($n$ = 68–75 embryos/condition, $n$ = 8–9 litters/condition) showed that the maternal enriched diet alone did not significantly influence overall embryo morphometric measurements (Figure 2, Tables S2 and S3). In EtOH-exposed embryos, the maternal enriched diet led to lower body weight and shorter crown-rump distance (Figure 2A,G, Tables S2 and S3) compared to the maternal standard diet, but either measurement did not significantly differ from controls. Ethanol exposure of embryos subjected to the maternal standard diet led to significantly higher brain weight, longer head height, and longer head width (Figure 2B,D,E, Tables S2 and S3). The maternal enriched diet significantly safeguarded embryo head width against the effects of ethanol exposure but had no beneficial impact on embryo brain weight or head height. In both maternal diet conditions, ethanol exposure did not affect embryo body weight, brain to body weight ratio, head length, crown-rump distance, distance between eyes, or placenta weight (Figure 2A,C,F–I, Tables S2and S3).

To further explore if a methyl donor-enriched maternal diet could have a protective effect against early fetal alcohol exposure, we performed a detailed assessment of visible morphological defects in late-gestation embryos (E18.5) (Figure 3A–C). The types of defects included growth restriction (Figure 3C-III, XI, XII), eye defects (e.g. anophthalmia, microphthalmia) (Figure 3C-VII), brain anomalies (e.g. forebrain or midbrain malformations), and other defects (e.g. body dysmorphism, polydactyly; Figure 3C-IV, VIII). The overall frequency of morphological defects was markedly highest in EtOH-exposed embryos subjected to a maternal standard diet (Figure 3A, Table S4), which also displayed a broader frequency distribution among the different types of defects (Figure 3B, Table S4). Interestingly, early fetal alcohol exposure was particularly linked to brain malformations, which were exclusively identified in EtOH-exposed embryos, and to growth restriction, which was prevalent in EtOH-exposed embryos for both the standard and enriched maternal diet conditions (Figure 3B, Table S4). Compared to the standard maternal diet, the enriched maternal diet led to a considerably lower overall frequency of morphological defects in EtOH-exposed embryos (STD-EtOH: 0.19, ENR-EtOH: 0.07) (Figure 3A,

**FIGURE 2** Maternal methyl donor supplementation alleviates late-gestation abnormal morphometrics induced by early fetal alcohol exposure. Morphometric measurements of E18.5 embryos subjected to a maternal standard diet (control; $n$ = 68, ethanol-exposed; $n$ = 70) or a maternal methyl donor-enriched diet (control; $n$ = 69, ethanol-exposed $n$ = 75). Statistical analysis: Shapiro–Wilk test followed by either non-parametric tests (Kruskal–Wallis test and Pairwise Wilcoxon Rank Sum tests) or parametric tests (Levene's test and one-way anova with pairwise comparisons and, if required, Welch's correction) with Benjamini–Hochberg *p*-value adjustment. *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$. See associated Tables S2 and S3.

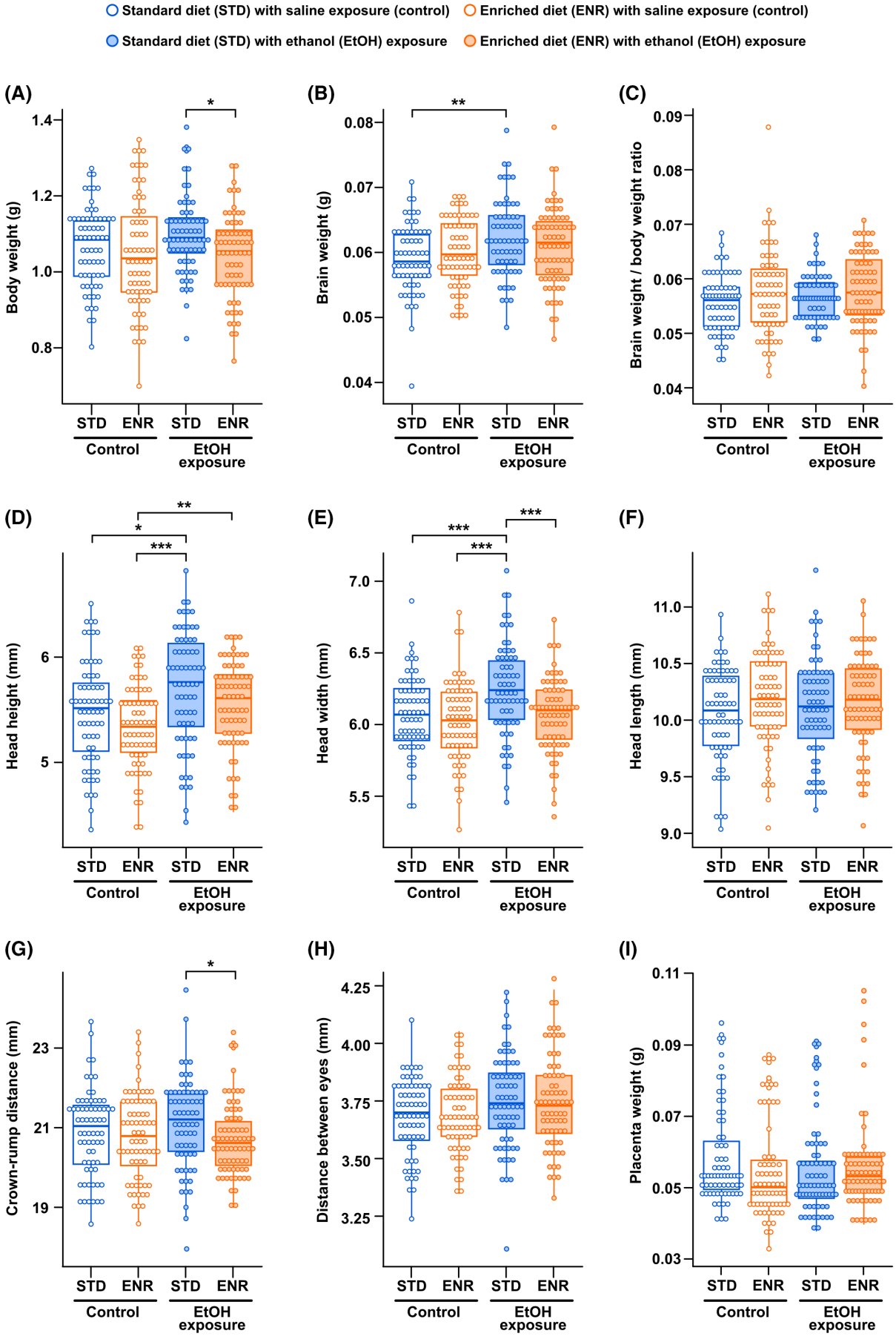

Standard diet (STD) with saline exposure (control)
Enriched diet (ENR) with saline exposure (control)
Standard diet (STD) with ethanol (EtOH) exposure
Enriched diet (ENR) with ethanol (EtOH) exposure
(A)
Body weight (g)
1.4
1.2
1.0
0.8
*
(B)
Brain weight (g)
0.08
0.07
0.06
0.05
0.04
**
(C)
Brain weight / body weight ratio
0.09
0.08
0.07
0.06
0.05
0.04
(D)
Head height (mm)
6
5
**
*
***
(E)
Head width (mm)
7.0
6.5
6.0
5.5
***
***
(F)
Head length (mm)
11.0
10.5
10.0
9.5
9.0
(G)
Crown-rump distance (mm)
23
21
19
*
(H)
Distance between eyes (mm)
4.25
4.00
3.75
3.50
3.25
(I)
Placenta weight (g)
0.11
0.09
0.07
0.05
0.03
STD
ENR
STD
ENR
Control
EtOH exposure

Table S4). The maternal enriched diet also eliminated certain types of ethanol-induced defects; only brain malformations and growth restriction were observed in EtOH-exposed embryos (Figure 3B, Table S4). To account for possible maternal effects in response to the various prenatal environmental conditions, we next examined the frequency of embryos with morphological defects per litter (Figure 3D, Table S5). The maternal enriched diet led to a lower proportion of affected litters compared to the maternal standard diet in both control (STD-Ctl: 3/8 vs. ENR-Ctl: 1/8) and ethanol exposure (STD-EtOH: 5/9 vs. ENR-EtOH: 3/9) groups. When looking at the range of frequencies of embryos with morphological defects in the affected litters, ethanol exposure led to more severely affected litters compared to controls for both maternal diets, but to a more moderate extent for the enriched diet (STD-Ctl: 0.10–0.13, ENR-Ctl: 0.17, STD-EtOH: 0.17–0.43, ENR-EtOH: 0.20–0.27) (Table S5).

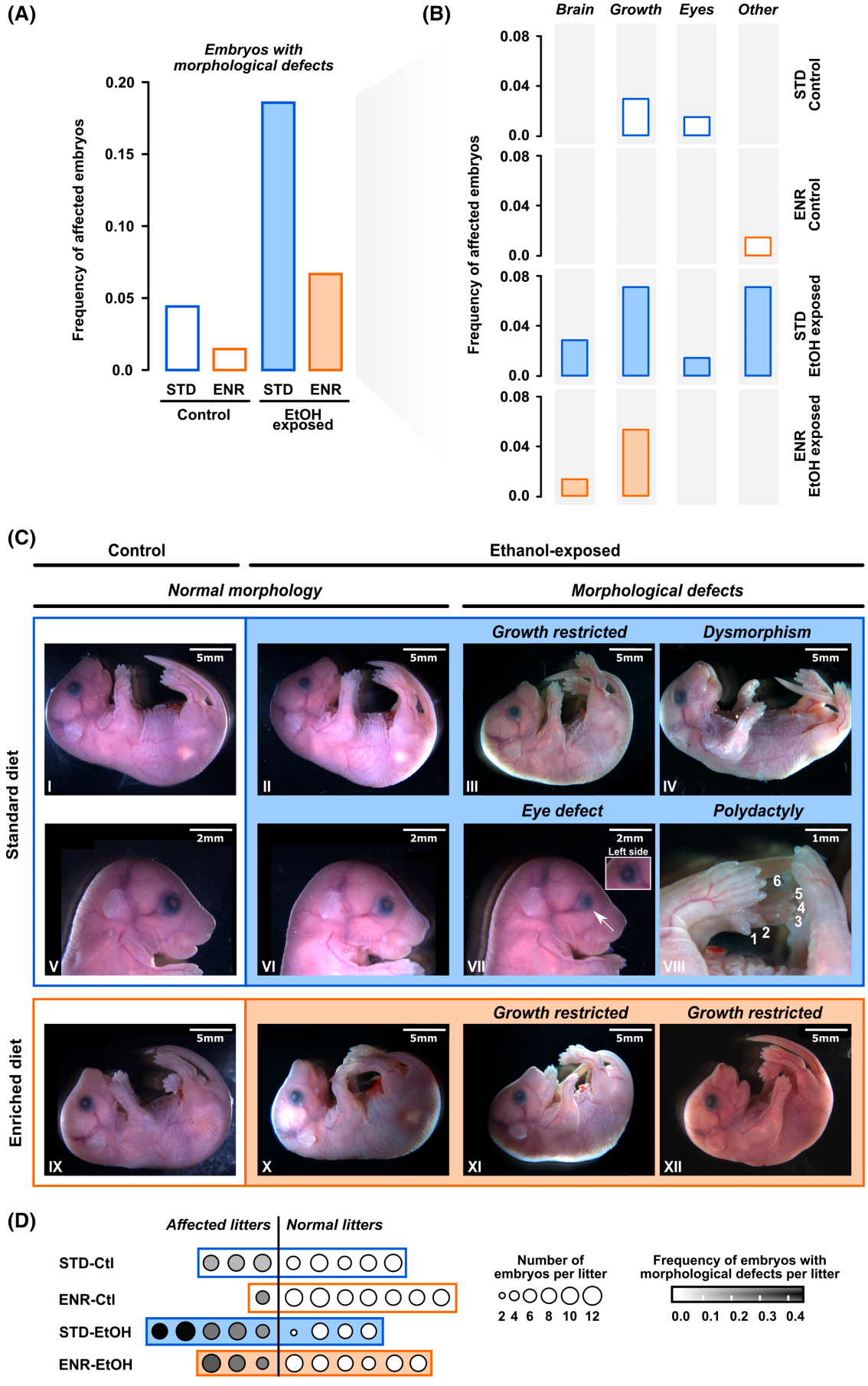
## 3.2 | Effect of early fetal alcohol exposure on skeletal development

Studies have shown that maternal alcohol intake during pregnancy can hinder skeletal development leading to ossification delays and craniofacial malformations in fetuses and offspring.[106,107,110,111] Therefore, we investigated if such delays and malformations would be visible at late gestation (E18.5) following early fetal alcohol exposure, if they would be particularly linked to the growth restriction phenotype prevalently observed in EtOH-exposed E18.5 embryos, and if a maternal diet enriched in methyl donors would have a protective effect on skeletal development. To do so, we performed skeletal staining of E18.5 morphologically normal embryos (control and EtOH-exposed) and growth-restricted EtOH-exposed embryos subjected to either the standard or the enriched maternal diet and completed a thorough analysis of primary ossification and craniofacial structure (Figure 4A–C, Table S6). The selection of growth-restricted EtOH-exposed E18.5 embryos was based on their abnormal stunted morphology and significantly lower body weight (Figure 4D, Tables S7 and S8). For all samples, we did not observe abnormal vertebral body count, limb defects, bone deformities, severe craniofacial structural anomalies or generalized skeletal dysplasia. We did however detect ossification delays exclusively in EtOH-exposed embryos, most predominantly in growth-restricted embryos for both maternal diets (Figure 4A, Table S6). All growth-restricted EtOH-exposed embryos under the standard maternal diet had ossification delays (7/7, frequency = 1.00) whereas a slightly lower frequency of ossification delays was observed in growth-restricted EtOH-exposed embryos under the enriched maternal diet (5/6, frequency = 0.83) (Figure 4A, Table S6). Morphologically normal EtOH-exposed embryos under both maternal diets showed much lower frequencies of ossification delays (STD-EtOH (normal): 1/11, frequency = 0.091; ENR-EtOH (normal): 1/10, frequency = 0.10) (Figure 4A, Table S6). We observed delayed ossification of the occipital bone (Figure 4B,C-III, XII) and 5th sternebrae (Figure 4B,C-VI, XV), with several EtOH-exposed embryos showing delays in both (Table S6). Growth-restricted EtOH-exposed embryos also had mild craniofacial hypoplasia demonstrated by significantly shorter head and snout length compared to morphologically normal control and EtOH-exposed embryos (Figure 4C-IX, XVIII, E, F, Tables S7 and S8). Overall, the maternal methyl donor-enriched diet had little to no effect in preventing skeletal abnormalities associated with early fetal alcohol exposure, which were primarily linked to the ethanol-induced growth restriction phenotype.

## 3.3 | Effect of early fetal alcohol exposure on imprinted DNA methylation and gene expression

It has been well established that DNA methylation patterns must be faithfully maintained in imprinting control regions (ICRs) during early embryonic development[93,95,96] and that alcohol exposure as well as methyl donor intake (deficiency or excess) can interfere with these mechanisms.[30,32,33,35] Since many imprinted genes are involved in embryonic and placental growth regulation,[103–105] we questioned whether the growth restriction phenotype

**FIGURE 3** Maternal methyl donor-enriched diet protects embryos from late-gestation morphological defects associated with early fetal alcohol exposure. Total frequency of morphological defects (A) and of the specific types of morphological defects (growth restriction, brain anomalies, eye defects, and other defects) (B) in E18.5 embryos subjected to a maternal standard diet (control; $n = 68$, ethanol-exposed; $n = 70$) or a maternal methyl donor-enriched diet (control; $n = 69$, ethanol-exposed $n = 75$). See associated Table S4. Statistical analysis (A): pairwise chi-squared tests with Yates continuity correction $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$. (C) Images of E18.5 morphologically normal embryos and embryos with various morphological defects associated with ethanol exposure for both maternal diet conditions. (D) Comparison of the number of affected litters, number of E18.5 embryos per litter, and frequency of E18.5 embryos with morphological defects per litter between the four conditions. See associated Table S5.


(A)
Embryos with morphological defects
Frequency of affected embryos
0.20
0.15
0.10
0.05
0.0
STD
ENR
STD
ENR
Control
EtOH exposed
(B)
Brain
Growth
Eyes
Other
Frequency of affected embryos
0.08
0.04
0.0
STD Control
ENR Control
STD EtOH exposed
ENR EtOH exposed
(C)
Control
Ethanol-exposed
Normal morphology
Morphological defects
Standard diet
Growth restricted
Dysmorphism
Eye defect
Polydactyly
Left side
Enriched diet
Growth restricted
Growth restricted
5mm
2mm
1mm
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
(D)
Affected litters
Normal litters
STD-Ctl
ENR-Ctl
STD-EtOH
ENR-EtOH
Number of embryos per litter
2 4 6 8 10 12
Frequency of embryos with morphological defects per litter
0.0 0.1 0.2 0.3 0.4

induced by early fetal alcohol exposure could be linked to dysregulation of ICR DNA methylation and expression of imprinted genes and, if so, whether the maternal methyl donor-enriched diet would have a protective effect. Using a targeted approach (*Implicon*[127]) that enables the detection of DNA methylation at single-CpG resolution within a 250–500 bp DNA segment, we examined two regions within the *H19* ICR associated with the *H19-Igf2* locus[129] (i.e., *H19* ICR-A, ICR-B) and one ICR associated with *Igf2r*,[130] three imprinted genes with well-defined growth regulatory roles during early development, as well as several other ICRs associated with *Gpr1-Zdbf2*, *Nesp*, *Gnas*, *Mest/Peg1*, *Snrpn*, and *Commd1-Zrsr1*. We compared ICR DNA methylation (Figure 5A, Figures S3 and S4) as well as expression levels (Figure 5B–D, Figure S5) among E18.5 morphologically normal control and EtOH-exposed embryos, and growth-restricted EtOH-exposed embryos subjected to either the standard or the enriched maternal diet. Neither *H19* nor *Igf2r* ICRs showed any significant differences in single-CpG DNA methylation levels (Figure 5A, Figure S3A) or in average ICR DNA methylation levels (Figure S3B) among the different conditions. There were also no significant differences in average DNA methylation levels for the other six ICRs we examined (Figure S4). For all embryos, DNA methylation levels of all ICRs were within a standard range of 45%–55%. Although no changes in DNA methylation were detected, growth-restricted EtOH-exposed embryos subjected to the maternal enriched diet had a slight increase in *H19* and *Gnas* expression (~1.3-fold compared to STD-Ctl) (Figure 5B, Figure S5D). No significant differences in gene expression were detected for any other imprinted gene (Figure 5C,D, Figure S5A–C,E–H). Altogether, neither early fetal alcohol exposure nor the maternal methyl donor-enriched diet had any major impact on DNA methylation at these specific ICRs or on gene expression of associated imprinted genes in forebrain of late-gestation embryos, even in those with an ethanol-induced growth restriction phenotype.

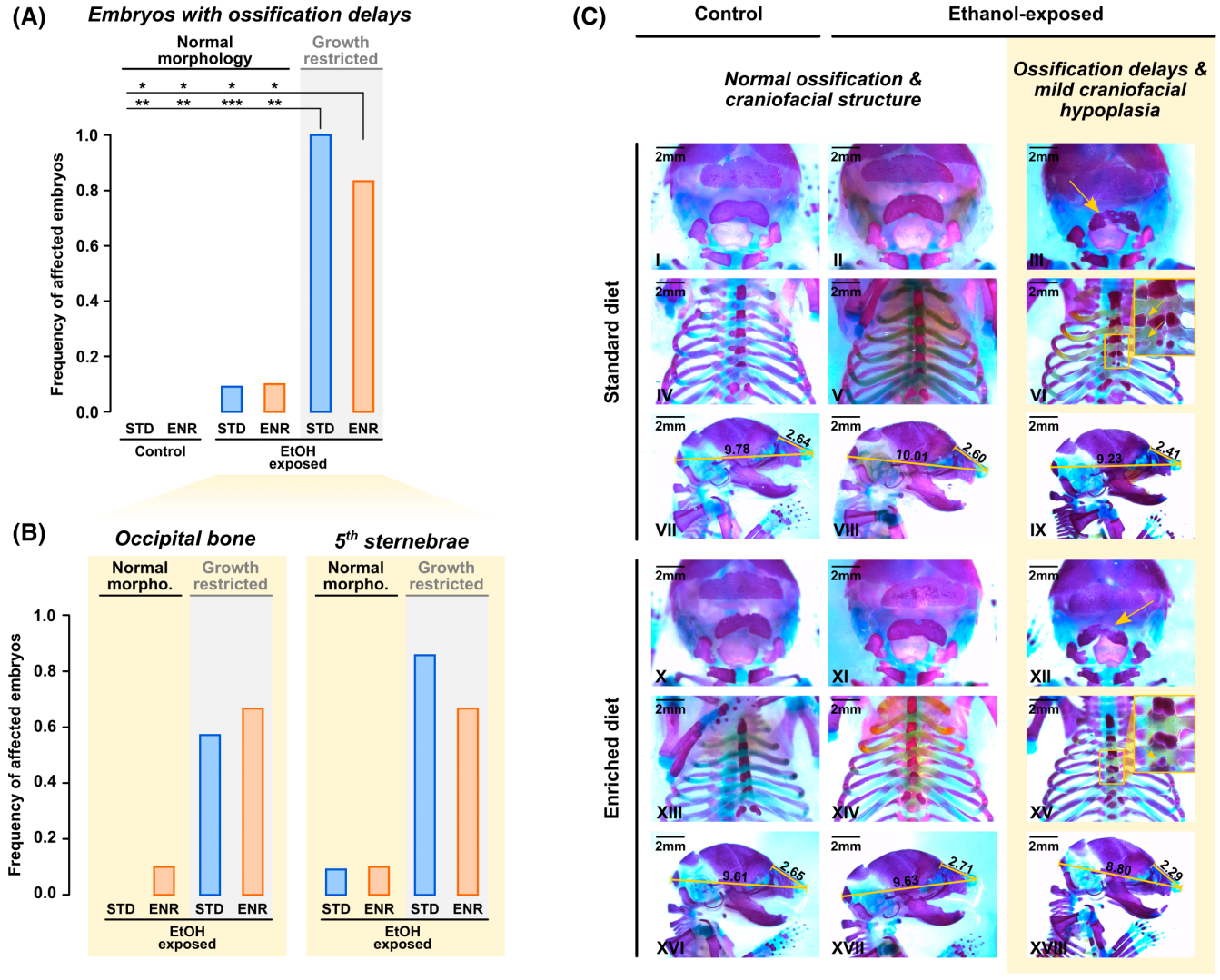
# 4 | DISCUSSION

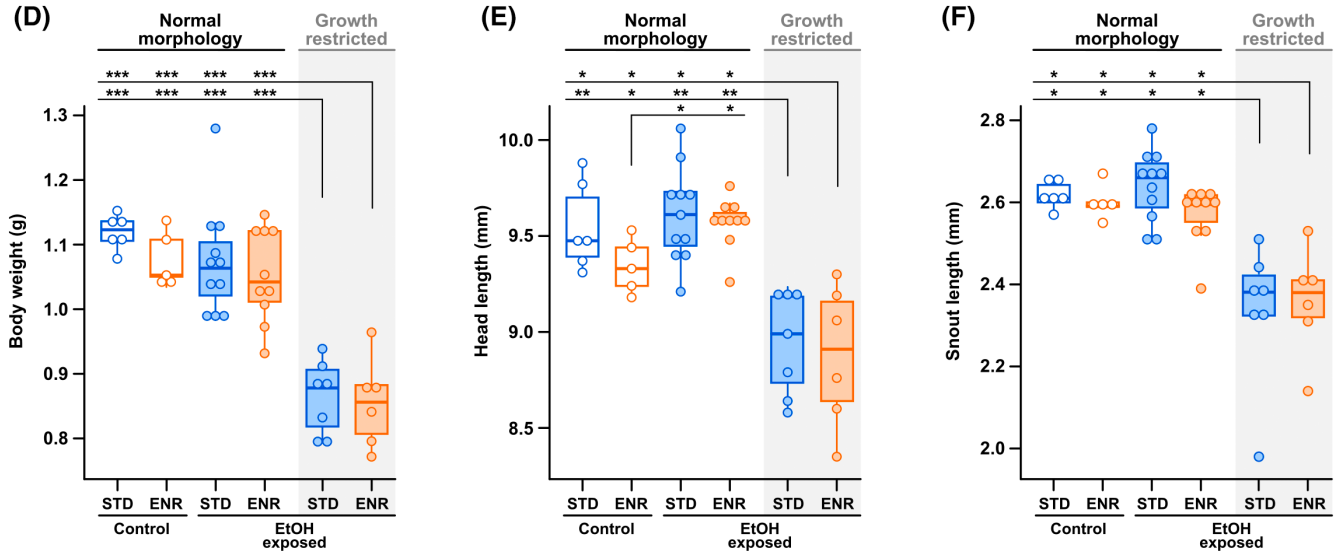
It is now recognized that fetal alcohol exposure during any gestation period can have serious teratogenic effects on embryo development and lead to FASD,[1–5,14] yet the impact of alcohol exposure during the preimplantation period, a particularly vulnerable developmental window,[83] remains considerably understudied. Although FASD is a worldwide public health burden,[12,131] there are no tangible measures to proactively mitigate the effects of fetal alcohol exposure prior to birth. One way to prevent or attenuate the related adverse developmental outcomes could be through maternal diet interventions using methyl donors.[110–118] Here, we demonstrate that exposure to acute levels of alcohol during preimplantation (E2.5) leads to long-term FASD-like phenotypes in late-gestation embryos (E18.5), and show that moderate enrichment of folic acid, choline, betaine, and vitamin B12 in the maternal diet prior to conception and throughout gestation has a protective effect, significantly reducing the incidence and severity of developmental defects in ethanol-exposed embryos.

## 4.1 | Lasting developmental impact of early preimplantation alcohol exposure

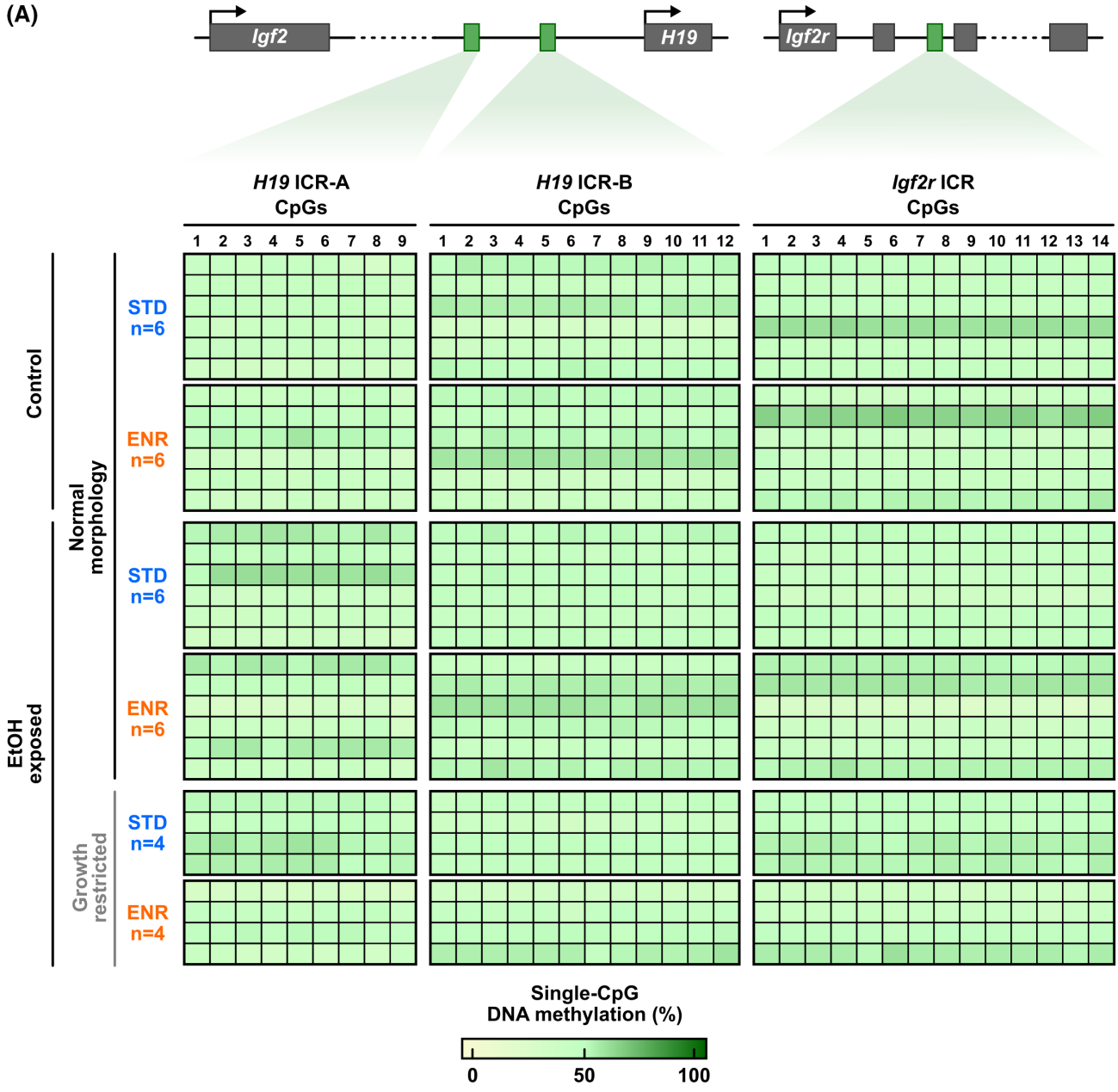
A growing body of evidence substantiates that the preimplantation embryo (i.e., fertilization to E4.5 in mice, E7-9 in humans) is vulnerable to alcohol teratogenicity,[30,33,106–109] refuting the outdated all-or-nothing premise that preimplantation alcohol exposure leads to embryos either surviving unharmed or dying during early gestation. Here, acute ethanol exposure at E2.5 (i.e., two doses of 2.5 g/kg with a 2-h interval) led to a variety of morphological malformations at late gestation (E18.5), including growth restriction, brain anomalies (e.g. forebrain or midbrain malformations), eye defects (e.g. anophthalmia, microphthalmia), and other defects such as polydactyly and

**FIGURE 4** Growth restriction linked to early fetal alcohol exposure is accompanied by ossification delays and mild craniofacial hypoplasia in both standard and enriched maternal diet conditions. Total frequency of ossification delays (A) and of occipital bone and 5th sternebrae ossification delays specifically (B) in E18.5 embryos with normal morphology (control and ethanol-exposed) and with ethanol exposure-associated growth restriction in both standard and enriched maternal diet conditions. See associated Table S6. Statistical analysis (A): pairwise chi-squared tests with Yates continuity correction. (C) E18.5 embryo skeletal staining images showing examples of normal ossification, ossification delays (occipital bone (III, XII), 5th sternebrae (VI, XV)), and mild craniofacial hypoplasia (shorter head and snout length (IX, XVIII)). (D–F) Body weight, head length, and snout length of E18.5 embryos stained for skeletal analysis. See associated Tables S6 and S7. Statistical analysis (D–F): Shapiro–Wilk test followed by either non-parametric tests (Kruskal–Wallis test and pairwise Wilcoxon rank sum tests) or parametric tests (Levene's test and one-way anova with pairwise comparisons and, if required, Welch's correction) with Benjamini–Hochberg *p*-value adjustment. Number of embryos: standard maternal diet; control (normal morphology: $n = 6$) and ethanol-exposed embryos (normal morphology: $n = 11$, growth-restricted: $n = 7$), enriched maternal diet; control (normal morphology: $n = 5$) and ethanol-exposed embryos (normal morphology: $n = 10$, growth-restricted: $n = 6$). $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$.




body dysmorphism, as well as higher brain weight and larger head dimensions (i.e., head height and width). The frequency (19% of embryos) and types of late-gestation malformations were highly similar to what we reported at midgestation (E10.5) in a previous study using the same exposure model,[30] suggesting that, in affected embryos,


(A)
Igf2
H19
Igf2r
H19 ICR-A CpGs
H19 ICR-B CpGs
Igf2r ICR CpGs
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Control
EtOH exposed
Normal morphology
Growth restricted
STD n=6
ENR n=6
STD n=6
ENR n=6
STD n=4
ENR n=4
Single-CpG DNA methylation (%)
0
50
100

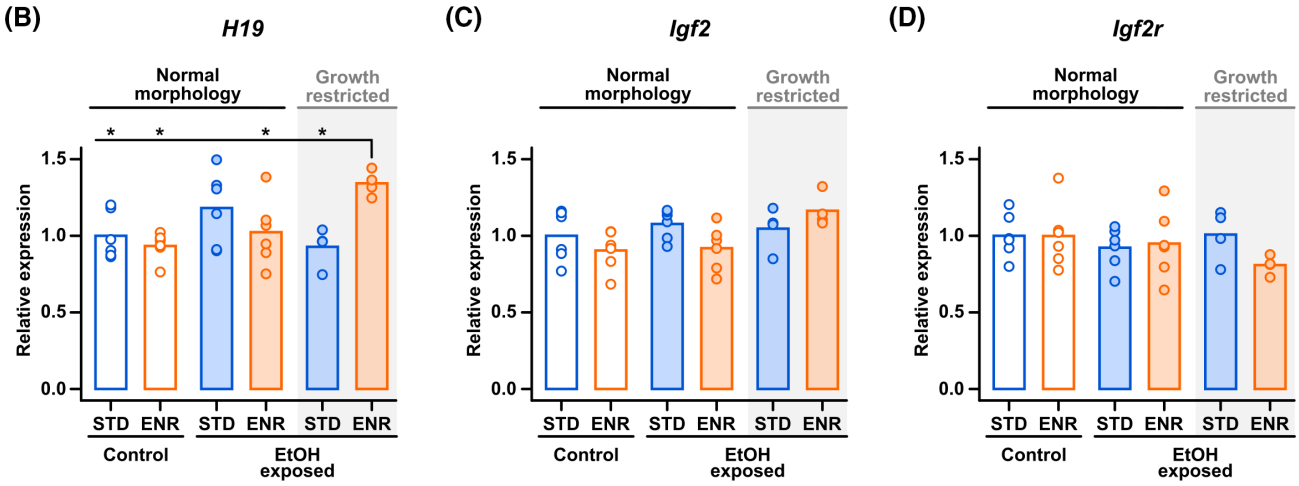

(B)
H19
(C)
Igf2
(D)
Igf2r
Normal morphology
Growth restricted
Relative expression
0.0
0.5
1.0
1.5
STD ENR STD ENR STD ENR
Control
EtOH exposed

**FIGURE 5** Early fetal alcohol exposure does not affect DNA methylation at the *H19* and *Igf2r* ICRs in forebrain of late-gestation embryos. (A) DNA methylation levels of single-CpGs in two regions of the *H19* ICR (*H19* ICR-A/B) and at the *Igf2r* ICR in E18.5 morphologically normal embryos (control or EtOH-exposed) and growth-restricted EtOH-exposed embryos subjected to a standard (STD) or enriched (ENR) maternal diet. See associated Figure S3. (B–D) Relative expression levels (normalized to *Hprt1* and STD-Control) of *H19*, *Igf2*, and *Igf2r*, respectively, in same embryos used for DNA methylation analysis. Gene expression statistical analysis (B–D): Shapiro–Wilk test followed by Levene's Test and one-way anova with pairwise comparisons, Welch's correction (if required), and Benjamini–Hochberg *p*-value adjustment. *$p \leq .05$. Number of embryos: Standard maternal diet: control (normal morphology: $n = 6$) and ethanol-exposed embryos (normal morphology: $n = 6$, growth-restricted: $n = 4$); Enriched maternal diet: control (normal morphology: $n = 6$) and ethanol-exposed embryos (normal morphology: $n = 6$, growth-restricted: $n = 4$).

the adverse developmental effects of acute alcohol exposure during preimplantation are prompt and persist over time. Higher-dose acute ethanol exposure during preimplantation can dramatically increase the severity of morphological malformations and the proportion of affected embryos. For instance, in a pioneer study where mouse dams were treated with a single acute dose of 5.8 g/kg ethanol at E2, there was a high incidence of fetal death occurring prior to E15 and severe malformations at E14 (e.g. hydrocephaly, craniofacial hypoplasia, delayed palatal closure, renal hypoplasia, exomphalos, severe growth restriction) were observed in 67%–100% of ethanol-exposed embryos, with a considerable proportion of embryos having a combination of several malformations.[106] In our model, ~80% of ethanol-exposed embryos were morphologically normal at mid- and late gestation, appearing to have either escaped or self-restored the damage inflicted by alcohol toxicity. Though it is also possible that, despite being physically normal, neurobehavioral function in these embryos could be affected, which would only become apparent after birth. Although highly debilitating, physical defects only account for a small minority of FASD symptoms; children with FASD frequently have so-called invisible symptoms such as intellectual, behavioral, and emotional disorders, which only manifest in childhood and adolescence.[132–134] At midgestation, we showed that forebrain of both morphologically normal and abnormal embryos exposed to alcohol during preimplantation had DNA methylation alterations in genomic regions linked to neurodevelopmental processes accompanied by some associated gene expression dysregulation,[30] which could eventually lead to defective cerebral cortex development and function, a main cause of neurodevelopmental and neurological disorders.[135–137]

As with postimplantation alcohol exposure models, it is becoming clear that the alcohol exposure paradigm during preimplantation greatly influences teratogenesis. However, a prevalent developmental defect observed in fetal alcohol exposure studies regardless of dosage, duration, or developmental timing is intrauterine growth restriction,[106,107,110,111] which can be associated with long-term physical ramifications, including skeletal malformations.[138–141] Although mammalian ossification of skeletal structures only begins at mid- to late gestation[142] (e.g. E13-14 in mice) and studies identify gastrulation through neurulation (i.e., E6.25–E10 in mice) as a key developmental window for alcohol-induced alteration of craniofacial development,[143] both preimplantation and postimplantation fetal alcohol exposure have been shown to hinder skeletal development.[106,107,110,111] In mouse models, acute high-dose ethanol exposure (5.8 g/kg) at E2[106] or E9,[110] chronic moderate-dose ethanol exposure (2.9 g/kg) from E0.5 to E8.5[107] and chronic high-dose ethanol exposure (5.0 g/kg) from E6 to E15[111] led to growth restriction and to a variety of long-term skeletal malformations ranging in severity (e.g. ossification delays, craniofacial abnormalities, vertebral anomalies, limb malformations). Here, our results further support that fetal alcohol exposure during preimplantation can have lasting adverse effects on skeletal development (i.e., ossification delays and mild craniofacial hypoplasia), and show a strong correlation between skeletal delays and alcohol-induced growth restriction.

On a molecular level, epigenetic dysregulation of imprinted genes has been examined as a potential mechanism of intrauterine growth restriction,[102,144] particularly the *H19-Igf2* locus[145–147] as *H19* suppresses,[103] while *Igf2* promotes[104] placental and fetal growth. In a Japanese rice fish model of fetal alcohol exposure during the first 48 h of development, ethanol-exposed embryos had decreased expression of DNA methyltransferase 1,[148] the enzyme responsible for maintaining DNA methylation at imprinting control regions (ICRs) in early embryonic development,[93,96,149] which could lead to permanent loss of DNA methylation at ICRs altering expression patterns of associated imprinted genes. *H19-Igf2* ICR DNA methylation (i.e., *Igf2* ICR0-3 and *H19* ICR)[129] and transcriptomic dysregulation, often in a tissue-specific manner, has been observed in various animal models of fetal alcohol exposure.[33,35,109] For instance, E16.5 mouse embryos chronically exposed to ethanol (7.25 g/kg) from E0.5 to E15.5 had tissue-specific loss of DNA methylation at *Igf2* ICR1, *Igf2* ICR2, and *H19* ICR in the heart, brain, and placenta, respectively, and increased *Igf2* expression in the brain.[35] In another mouse model, chronic ethanol exposure (2.9 g/kg) from E0.5 to E8.5 induced *H19* upregulation and downregulation in embryonic and placental tissues,

respectively, and *Igf2* downregulation only in the placenta at E9.5 and E16.5, but without any associated ICR DNA methylation alterations.[109] In the present study, growth restriction in embryos exposed to ethanol was not associated with DNA methylation or transcriptomic dysregulation of the *H19-Igf2* locus, or of several other imprinted loci (*Igf2r*, *Gpr1-Zdbf2*, *Nesp*, *Gnas*, *Mest/Peg1*, *Snrpn*, and *Commd1-Zrsr1*), except for a subtle (1.3-fold) increase in *H19* and *Gnas* expression, in fetal forebrain tissue at late gestation. However, we cannot rule out epigenetic dysregulation of *H19-Igf2*, or other imprinted loci as a potential mechanism of alcohol-induced growth restriction. In our previous study, using rRRBS (rapid reduced representation bisulfite sequencing), we observed partial loss of DNA methylation (>10% methylation difference, 100 bp tiles) at several ICRs in forebrain of ethanol-exposed embryos at midgestation.[30] As the fetal forebrain matures from mid- to late gestation, the cell-type composition is much more diversified,[150] therefore, perhaps DNA methylation dysregulation by alcohol is cell-type specific. With recent advances in low-input whole-genome bisulfite sequencing and single-cell approaches, a broader investigation of DNA methylation profiles subsequent to the time of exposure and at multiple developmental time points would be better suited to provide a more comprehensive conclusion. Moreover, since we and others have shown sex-dependent DNA methylation alterations associated with fetal alcohol exposure,[30,151,152] increasing the number of samples would enable methodical sex-specific analyses.

## 4.2 | Mitigating the effects of early fetal alcohol exposure with a maternal diet enriched in methyl donor nutrients

Maternal nutritional interventions using methyl donor-enriched diets have become increasingly promising for mitigating fetal alcohol exposure.[110–118] For instance, in a mouse (CD-1) model of chronic fetal ethanol exposure (5.0 g/kg/day; E6–E15), maternal high-dose folic acid (60.0 mg/kg; E1–E16) and vitamin B12 (1.0 mg/kg) combined supplementation mitigated developmental ethanol toxicity by reducing the incidence of skeletal abnormalities (E18).[111] However, similarly to how genetic differences can influence alcohol teratogenicity,[23–26] genetics may also contribute to the effectiveness of nutritional interventions during pregnancy. For example, supplementing only folic acid in the maternal diet (60.0 mg/kg; E1–E6) was able to prevent ethanol- (5.0 g/kg/day; E6–E15) induced defects in Balb/c mice,[112] but not in CD-1 mice.[111] Most studies so far have used maternal diets supplemented with only one or two methyl donor nutrients at high concentration, which could potentially cause adverse effects in offspring due to metabolic imbalance or excess supplementation.[119–123] Here, we showed that moderate supplementation of several methyl donor nutrients (folic acid: 5 mg/kg, choline: 5.76 g/kg, betaine: 5 g/kg, and vitamin B12: 0.5 mg/kg) in the maternal diet prior to conception and throughout gestation was able to safeguard embryo head width, substantially reduce the incidence of morphological defects as well as eliminate specific types of morphological defects (e.g. dysmorphism, polydactyly, eye defects) in mouse embryos (C57BL/6) exposed to acute levels of ethanol during preimplantation (E2.5). Another multinutrient maternal supplementation intervention has also been shown to successfully mitigate the adverse effects of postimplantation acute fetal alcohol exposure (EtOH 5.8 g/kg at E9; C57BL/6) (e.g. lower prenatal mortality rate, improved prenatal growth, drastic reduction skeletal malformations) in late-gestation embryos (E18).[110] However, their maternal methyl donor-enriched diet (3SZM chow), in comparison to ours, had three-fold higher levels of folic acid (15 mg/kg), choline (15 g/kg), betaine (15 g/kg) and vitamin B12 (1.5 mg/kg), and had additional supplementation of zinc (150 mg/kg) and L-methionine (7.5 g/kg). This same 3SZM diet fed to pregnant yellow agouti ($A^{vy}$) females, in which expression of the *agouti* gene and coat color phenotype is driven by DNA methylation of a long terminal repeat (LTR), led to increased DNA methylation at the *agouti* LTR and altered coat color phenotype in pups,[121] indicating that excess supplementation of methyl donors can impact DNA methylation profiles. Thus, although we and others have demonstrated the advantages of methyl donor nutrient supplementation in the maternal diet for mitigating fetal alcohol exposure on a morphological level, we remain cautious about its long-term impact on the embryonic epigenetic program. Sex-, tissue-, and cell-specific genome-wide analyses would be imperative to fully assess the impact of increased methyl donor nutrient levels in the maternal diet on the fetal methylome.

There have also been some studies in which postnatal nutritional interventions were used to mitigate the effects of fetal alcohol exposure. In animal models, memory and learning impairments in offspring exposed to alcohol during fetal development were moderately alleviated by postnatal choline supplementation,[153–155] and in humans, choline supplementation improved cognitive performance of children with FASD to some extent.[156,157] Overall, results of postnatal nutritional intervention studies are generally less conclusive than prenatal studies. The damage inflicted by fetal alcohol exposure may be too advanced after birth to be reversed by nutrient supplementation alone. Alternatively, the mechanisms driving the protective effect of methyl

donor nutrients may occur mostly in the mother; perhaps methyl donors act on minimizing alcohol toxicity prior to it reaching the embryo rather than on correcting the aftermath.

## 5 | CONCLUSION

Although the best way to prevent FASD remains to abstain from alcohol use during pregnancy, the results of this study suggest that supplementation of methyl donor nutrients in the maternal diet before and throughout pregnancy could be a potential proactive measure for FASD prevention, especially in cases where the mother has nutritional deficiencies, which could worsen the teratogenic effects of alcohol.[67,68] FASD and methyl donor nutrient deficiencies among pregnant women are particularly prevalent in lower income countries,[12,58–60] for instance, in sub-Saharan Africa, where some regions have the highest prevalence of children and youth with FASD worldwide,[12] many women of reproductive age (≥15–49 years) are folate deficient.[61] Ultimately, our work emphasizes the dangers of alcohol consumption during early gestation and the need for proper preconception and prenatal nutrition.

### AUTHOR CONTRIBUTIONS

Mélanie Breton-Larrivée, Amanda J. MacFarlane, and Serge McGraw conceptualized the study. Mélanie Breton-Larrivée and Lisa-Marie Legault contributed to data acquisition. Mélanie Breton-Larrivée, Elizabeth Elder, and Alexandra Langford-Avelar participated in data analysis. Elizabeth Elder and Serge McGraw wrote the manuscript. All authors read and approved the final manuscript.

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### DATA AVAILABILITY STATEMENT

The DNA methylation data from this study have been submitted to GEO Expression Omnibus (GSE222974). All other data that support the findings of this study are available in the methods and supplementary material of this article.

### DISCLOSURES

The authors declare that they have no conflicts of interests.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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