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Review

MicroRNAs as potential biomarkers for assessing prenatal alcohol exposure: A narrative review analyzing differences between human and animal studies

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ABSTRACT

Fetal alcohol spectrum disorders constitute a range of physical and cognitive abnormalities in newborn infants that are caused by prenatal alcohol exposure. Children with fetal alcohol spectrum disorders have facial abnormalities, small eyes and head size, and prominent cognitive and behavioral deficits that can persist into adulthood. Almost half of such children born in the USA each year go undiagnosed. Some alcohol consumption during pregnancy is reported by approximately 10 % of women in the USA, with almost half of all pregnancies being unintended. Early identification of alcohol-affected children is essential for implementing effective interventions to mitigate the adverse secondary effects of alcohol exposure that may emerge later in life. Alcohol exposure can disrupt neurodevelopment of the fetus or infant via multiple mechanisms, leading to behavioral and cognitive alterations. MicroRNAs can serve as possible biomarkers of pre- or post-natal alcohol exposure. This review summarizes advances in the literature on microRNAs associated with alcohol exposure in humans and mice/rats. In the human studies, alterations in the expression levels of eight microRNAs in maternal blood serum in the first and second trimesters (miR-124–3p, miR-125b–5p, miR-132–3p, miR-134–5p, miR-138–5p, miR-302b–5p, miR-346, miR-9–5p) caused by maternal alcohol exposure could be potential biomarkers. Some similarities in microRNA expression were found in mouse/rat studies that assayed fetal and infant brain tissue samples. However, there was limited agreement with the human study using fetal brain tissue except for miR-9–5p. More human studies are needed to identify potential microRNA biomarkers in blood samples of women who have heavily consumed alcohol during pregnancy and their infants. Also, treating pregnant women and their infants with folate and choline when the women report they have heavily consumed alcohol during pregnancy could partially alleviate the adverse effects of alcohol in the infants.

1. Introduction

Prenatal alcohol (ethanol) exposure causes a range of physical and cognitive abnormalities in newborn infants that persist into adulthood[1,2] and are known as fetal alcohol spectrum disorders (FASDs). The estimated global prevalence of FASD among the general population is 7.7 cases/1000 individuals, ranging from 19.8 per 1000 in the WHO European Region to 0.1 per 1000 in the World Health Organization (WHO) Eastern Mediterranean Region.[3] Many women consume alcohol before knowing they are pregnant; however, FASDs cannot be diagnosed early in utero.[3,4] In a recent meta-analysis the global prevalence of alcohol use during pregnancy was 9.8 %.[5] A 2013 report found that ~18 % of pregnant women in the USA consumed alcohol, with 6.8 % reporting binge-drinking episodes,[6] which may markedly impair fetal development.[7] Furthermore, of the estimated 80,000 children born with FASDs each year in the USA, almost half are undiagnosed. Children with FASDs experience facial defects, small eyes and head size, and marked cognitive and behavioral deficits,[8] which can persist into adulthood. Fetal alcohol syndrome is the most severe pattern of FASDs and is diagnosed as a specific pattern of facial abnormalities, microcephaly, and growth retardation. However, it is only seen in a fraction of the estimated number of fetal alcohol-affected children.[8,9] Approximately 50 % of lactating women reported occasional alcohol use, and only 13 % received counseling from their healthcare provider about the risks of alcohol use during lactation.[10,11] In the USA, 56 % of infants are breastfed for at least 6 months.[12]

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FASD will continue to be a significant medical and societal burden with 10 % of women consuming some alcohol during pregnancy in the USA[13] and almost half of all pregnancies unintended.[14] FASD prevalence in the USA and some Western European countries is estimated at 2 %-5 % of school children.[15] Impaired learning and memory, language development, and abnormal social behavior are associated with FASD.[16] The social behavior changes in affected adolescents can resemble those typically associated with autism. Animal models of prenatal alcohol exposure also exhibit behavioral deficits such as decreased social investigation and play fighting, as well as decreased social motivation in late adolescence and adulthood.[17,18]

Effective intervention strategies require the identification of factors that can reduce the severity of FASD. Early identification of alcohol-affected children is paramount to facilitating early intervention, which can alleviate some of the adverse secondary effects of prenatal alcohol exposure that may emerge later in life.[19] However, it is difficult to identify alcohol-affected children, particularly those children who lack the characteristic dysmorphic facial features of fetal alcohol syndrome and partial fetal alcohol syndrome.[20] Moreover, it is very difficult to identify prenatal alcohol exposure-related dysmorphology in infancy and early childhood, when therapy could be particularly effective.[21]

Neurodevelopment of the fetus can be impaired by prenatal alcohol exposure via multiple mechanisms including cell proliferation, migration, differentiation, synaptogenesis, and myelination, depending upon the stage of development and other exposure parameters.[22–24] There may be lasting changes in cell function, altering gene expression[25,26] and compromising synaptic plasticity,[27,28] which contribute to the pathological changes in neural structure and function brought about by alcohol. Such changes lead to behavioral and cognitive alterations[29,30] that seriously impair the quality of life of those prenatally exposed to alcohol and their families.

Nutritional variables are included among several factors that can modify the teratogenic effects of alcohol.[31,32] Administration of choline has been shown to reduce the severity of alcohol's teratogenic effects, including physical,[33] neuropathological,[34,35] and behavioral outcomes.[34,36–38] Given during prenatal alcohol exposure, choline alleviates alcohol-related decreases in birth and brain weights, and working memory deficits. Importantly, choline can reduce the severity of fetal alcohol effects when administered postnatally after the alcohol exposure has occurred. At this time, choline targets alcohol's effects on behaviors associated predominately with hippocampal function,[34,36–39] including trace fear conditioning.[40]

Conventional imaging cannot diagnose FASDs early *in utero* so it is necessary to find early biomarkers that can predict which at-risk fetuses will go on to have FASD postnatally and provide guides to interventions that might prevent or ameliorate FASDs. Among potential biomarkers, microRNAs (miRNAs) have attracted attention.[41–43] MiRNAs are short (20–24 nts), single-stranded non-coding RNA molecules that regulate gene expression of their complementary mRNA targets.[44,45] MiRNAs play important roles in multiple biological processes, including cell cycle control, cell growth and differentiation, apoptosis, embryo development and brain development.[46–49] They have also been implicated in neurological diseases, and changes in miRNA levels have been reported in animal models of FASDs as well as in human fetuses exposed to alcohol *in vivo*.[50–55] Altered fetal brain levels of specific miRNAs might be used to predict FASDs, and fetal brain miRNAs have been detected in the maternal circulation.[56] Alcohol use during pregnancy caused alterations in serum miRNAs in pregnant women.[57,58] In this review, we have performed a PubMed literature search of miRNAs in human subjects and animal models exposed to alcohol to assess whether the levels in maternal or infant blood samples and fetal brain tissues could serve as potential biomarkers of the disorder. We have critically analyzed the findings for correspondence, or lack of, between the human and animal studies. In addition, medical interventions that were found to reverse some of the changes of alcohol exposure have been highlighted. Those miRNAs found to be potential biomarkers of prenatal alcohol exposure could be important in distinguishing this condition from, for example, autism spectrum disorder, attention deficit hyperactivity disorder, and could suggest possible interventions to prevent or retard progression of the disorder and improve the quality of life of affected individuals.

2. Search strategy

We performed a PubMed search for miRNAs using the search terms "microRNA" and "fetal alcohol disorder." A total of 56 studies on alcohol exposure in pregnant women and infants (as well as mice and rats) published January 2008 to May 2024 were retrieved. These studies compared alcohol exposure subjects with controls that were not exposed or only exposed to low doses of alcohol. Of the 39 selected articles, 2 were with stem cells or diabetic pregnancies, 1 used a chick model, 1 a monkey model, 1 a sheep model, and 9 were reviews. The present review involved 17 articles, including 6 studies in humans, 8 in mice, and 3 in rats (Fig. 1). We did not include 8 studies in mice, 1 in rats as they were not on miRNAs or 2 studies using zebrafish (2 of the mouse studies had also included zebrafish). Infants with FASD have facial abnormalities, small eyes and head size, and prominent cognitive and behavioral deficits,[8] and obtaining a detailed history of maternal alcohol consumption during pregnancy is important to making a diagnosis.

3. MicroRNAs in mothers and infants after alcohol consumption by mothers

3.1. Human studies

3.1.1. Maternal blood plasma

Salem et al.[59] recruited 34 HEa women (heavily alcohol-exposed mother with an FASD-affected child, gestational age at enrolment [18.3 ± 5.1] weeks), 23 HEua women (moderate to heavily alcohol-exposed mother with an apparently unaffected child, gestational age at enrolment [19.8 ± 5.1] weeks), and 36 UE women (low alcohol consuming or unexposed mothers with an unaffected child, gestational age at enrolment [18.0 ± 5.5] weeks). The plasma available for these

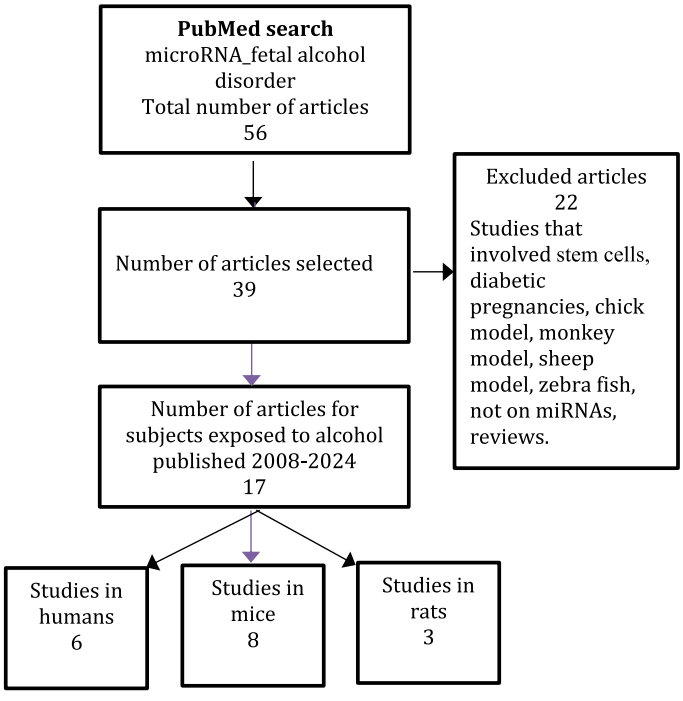

Fig. 1. Screening process for included literature.

pregnant women included second- and third-trimester plasma samples from 68 women who were profiled previously[57] (see below), and 25 third-trimester samples from newly assessed subjects.

There was no difference in cigarette use between the HEa and HEua women. Plasma samples were assessed for 752 miRNAs using miRNA miRNome PCR Panel. 153 miRNAs were expressed in at least 80 % of the samples in all the exposure groups. Several maternal miRNAs were identified in both HEa and HEua groups where no effect of prenatal alcohol exposure (relative to the UE group) was observed in the composite sample, but there was a significant effect of prenatal alcohol exposure when the samples were analyzed by gender. Significant 'alcohol-sensitive, fetal gender-specific' miRNAs in each group were identified. Male specific-alcohol sensitive second trimester: miR-361–5p, 328–3p, −146a−5p, −106b−5p, −25–3p, -let7c–5p, 151a3p, 320a, 223–5p. Male specific-alcohol sensitive third trimester: miR-let-7f-5p, 30a–5p, 6523p, 425–5p, 423–3p, 625–3p, 532–3p, 148a3p, 335–5p, -let-7c-5p, −126–5p, –125b–5p, 99a–5p. Female specific-alcohol sensitive second trimester: miR-454–5p, 1972, -26b–5p, 106b–3p, 143–3p, 199a–5p, 150–5p, 339–5p, 1245a. Female specific-alcohol sensitive third trimester: miR-329–3p, 320b, 2110, 125b–5p, −638.

A literature review was performed on miRNA levels in gestational pathologies caused by impaired placentation.[60] Placental and plasma levels of 8 of 11 $_{HEa}$miRNAs were significantly dysregulated in one or more of these gestational pathologies with expression of the majority of these 8 miRNAs altered in both fetal growth restriction and preeclampsia. An attempt was made to determine if circulating $_{HEa}$miRNA levels could explain the variance in gender and gestational age-adjusted neonatal height, weight, and head circumference, which are growth measures sensitive to *in utero* environment.[61] Eight of the $_{HEa}$miRNAs each significantly explained between 7 % and 19 % of infant variation in these growth measures (trimester 2: miR-299–3p,–491–3p,–885–3p, –518f–3p, 204–5p, 519a–3p; trimester 3: 222–5p, 449a, 204–5p). Furthermore, seven of the $_{HEa}$miRNAs were also associated with fetal growth restriction and preeclampsia as indicated by the literature review. A multivariate statistical regression model including the levels of all 11 $_{HEa}$miRNAs explained a much greater proportion of infant variance, between 24 % and 31 % in all these growth measures than accounting for them individually.

Balaraman et al.[57] studied 22 HEa (gestational age at delivery [37.9 ± 2.5] weeks), 23 HEua (gestational age at delivery [40.0 ± 1.1] weeks) and 23 UE women (gestational age at delivery [40.0 ± 1.1] weeks). Pregnant women were selected for enrolment if they reported either frequent daily or weekly episodic (binge) drinking in the month around conception or the most recent month of pregnancy. Women were enrolled in the study on average between 17 and 19 weeks of pregnancy. Plasma samples were obtained from mothers at each of the two interview timepoints. Gestational age at birth, birth size and gender of the infant were recorded. Live-born infants subsequently received an examination for the physical features of FASD and for growth. At approximately 6 and 12 months of age, infants were evaluated for neurobehavioral performance. Plasma miRNA profiles were measured using RT-PCR arrays. While alcohol consumption differed significantly across groups, the HEa and HEua groups were not different from each other with respect to prenatal alcohol exposure. No evidence for erythrocyte miRNA contamination was found in all the samples, and no effect of alcohol exposure on human placental lactogen levels assessed near the end of the third trimester; therefore, placental damage is unlikely to account for altered maternal miRNA profiles. Eleven miRNAs (miR-222–5p, 187–5p, 299–3p, -518f–3p, 760, 671–5p, 449a, 204–5p, 519a–3p) exceeded a false discovery rate (FDR) of $\alpha = 0.05$ and a total of 21 that exceeded $\alpha = 0.1$ for the main effect of exposure condition. Eleven significantly altered miRNAs were increased in plasma samples from women in the HEa group at both mid- and late pregnancy compared to both the HEua and UE groups.

3.1.2. Maternal blood serum

Darbinian et al.[56] recruited 40 alcohol-consuming subjects (age [26.2 ± 2.2] years, gestational age [15.5 ± 1.3] weeks), and 40 controls (no alcohol, drugs or medication; age [22.3 ± 1.7] years, gestational age [15.2 ± 1.4] weeks). Fetal brain and maternal blood from subjects undergoing elective termination of pregnancy were collected. Human fetal brain and maternal serum total RNA was isolated. Human fetal brain-derived exosomes were isolated from maternal serum.[62] By microarray analysis, when comparing pregnant women who consumed alcohol with their individually matched unexposed controls, the alcohol-exposed group showed greatly reduced serum miRNA levels at the gestational age of 11.3 weeks, but then the levels increased greatly by 18.3 weeks. Nine miRNAs were assayed by qRT-PCR in 6 alcohol-exposed and 6 unexposed matched control sera and the corresponding fetal brain tissues. All but one miRNA (miR-509) showed significant changes in expression (miR-124–3p, 125b–5p, 132–3p, 134–5p, 138–5p, 302b–5p, 346, 9–5p). A general upregulation of miRNA levels was seen in the second trimester maternal serum compared to the first trimester for the control cases. Prenatal exposure to alcohol was associated with a decrease in this upregulation from 3.8-fold to 2.0-fold. By contrast, in alcohol-exposed fetal brains the 1.9-fold downregulation of miRNA levels seen in controls was changed to a 2.0-fold increase late in the second trimester. In the first trimester samples, screened miRNAs were upregulated 2.5–5.5-fold in the fetal brains and downregulated 3.5–6.5-fold in the maternal serum, compared to their matched unexposed controls. In the second trimester samples, alcohol-exposure had smaller effects in the opposite direction i.e., down-regulation in the fetal brain specimens and slight upregulation in the maternal serum. Fetal brain-derived exosomes were isolated from maternal serum and assayed by qRT-PCR for two alcohol-responsive miRNAs, miR-9 and miR-132. Twenty alcohol-exposed cases were compared with their individually matched unexposed controls with 10 matched pairs from the first trimester and 10 from the second trimester. Both miR-9 and miR-132 were downregulated in fetal brain-derived exosomes from pregnant mothers who consumed alcohol. In maternal serum, both miR-9–5p and miR-132–3p were downregulated in the first trimester and miR-132–3p tended to be upregulated in the second trimester. The situation regarding miR-9–5p in the second trimester was less clear.

Gardiner et al.[58] studied 14 alcohol-consuming subjects (age [29.1 ± 6.7] years, gestational age [38.2 ± 2.2] weeks at delivery (visit 2)), and 16 controls (no alcohol, age [25.7 ± 3.7] years, gestational age [37.7 ± 3.7] weeks at delivery). Self-reported measures of alcohol use were used to classify subjects into alcohol and control groups. Self-reported use of other substances was supplemented by urine drug screens. Blood was collected at enrolment (visit 1) and again upon admission for labor and delivery (visit 2). Serum was isolated from the whole blood. Total RNA, including miRNA, was extracted from serum collected at visit 2. 41 % of the subjects in the alcohol group had AUDIT ≥ 8, 21 % reported ≥ 2 binge-drinking episodes per week in the periconceptional period and 85 % admitted to at least 1 binge episode since the last menstrual period. All the controls reported abstinence from alcohol after the last menstrual period and tested negative on all five biomarkers (GGT, %dCDT, UEtG, UEtS, PEth-DBS). The use of drugs other than alcohol (i.e., marijuana, cocaine, OMT or other opioids, and tobacco) was prevalent; however, there were no significant differences between the groups for any of these. Low levels of free hemoglobin were found in all the serum samples and with no differences between the alcohol and control groups. By microarray analysis, with ANCOVAs adjusting by hepatitis C, tobacco, and marijuana use, 55 miRNAs were identified in serum that were significantly altered between the alcohol-consuming and control groups; of these, 46 miRNAs were elevated and 9 were reduced in the alcohol-consuming group. The top 10 miRNAs with increased fold changes in the alcohol group were miR-509–5p, 3119, 26a-2–3p, 1279, 4743, 4799–3p, 4657, 3942–3p, 3126–3p, 514b–5p. The top 4 miRNAs with decreased fold changes in the alcohol group were miR-125a-5p, 602, 126, -3180–3p. qRT-PCR was performed for four miRNAs (miR-509–5p, 4657, 542–3p, 602) with high fold changes and low FDR values. MiR-509–5p, 4657, 542–3p,

which were elevated in the microarray data, were also increased in the RT-qPCR data, and miR-602 was decreased in both data sets. Hierarchical clustering using only significantly altered miRNAs grouped the subjects into two clusters, with each cluster consisting mostly of either alcohol-consuming or non-alcohol-consuming patients. MiR-122*(-3p), 126, 216b, 221*(–5p), 3119, 3942–5p, 4704–3p, 4743, 514b–5p, 602 were the top 10 discriminators between the two groups.

3.1.3. Infant blood plasma

Mahnke et al.[55] recruited 37 prenatal alcohol exposure women (age at delivery [28.3 ± 6.1] years, gestational age at delivery [38.9 ± 1.9] weeks) and 31 controls (age at delivery [25.0 ± 4.9] years, gestational age at delivery [39.2 ± 1.9] weeks). The mothers of infants in the alcohol-exposed group reported consuming an average of 4.5 oz absolute alcohol/occasion across pregnancy on an average of 1–2 days per week. Cigarette and marijuana use did not differ between the two groups. Plasma samples were obtained from infants at 2 weeks and 6.5 months postpartum. No significant differences in free hemoglobin levels attributable to alcohol exposure or infant gender were found. MiRNAs were assayed by qPCR. Infants expressed ~14 % fewer unique miRNAs at 6.5 months than at 2 weeks. However, whereas the control infants exhibited a ~18 % decline in total RNA content between 2 weeks and 6.5 months, infants with prenatal alcohol exposure had elevated plasma RNA levels at 6.5 months. 148 miRNAs were detected in at least 80 % of the samples of either the alcohol-exposed or control group at 2 weeks or 6.5 months. At 2 weeks, expression levels of 18 miRNAs differed between the alcohol exposed and control groups. At 6.5 months, expression of 26 miRNAs differed between the two groups. At 2 weeks, 72 % of these prenatal alcohol exposure-responsive miRNAs were upregulated by prenatal alcohol exposure (miR-663b, 320d, 30c5p, 21–5p, 143–3p, 22–3p, 1972, 624–5p, 18a–3p, 92a–3p, 598–3p, 23a–5p, 30e–5p), while at 6.5 months, 92 % of the miRNAs were upregulated by prenatal alcohol exposure (miR-126–3p, 328–3p, 30c–5p, 24–3p, 193a–5p, 103a–3p, 92a–3p, 143–3p, 140–5p, 23b–3p, 125a–5p, 194–5p, 30b–5p, 148a–3p, 16–5p, 19b–3p, 423–5p, 101–3p, 27b–3p, 532–5p, 23a–3p, 223–3p, 125b–5p, 30a–5p). Previous research suggests that there are gender differences in the early presentation of FASD and some of the cognitive impairments associated with prenatal alcohol exposure.[63,64] Some large-effect-size differences emerged when miRNA expression data were analyzed by gender. For instance, at 6.5 months, miR-328–3p was significantly elevated in plasma samples of female infants with prenatal alcohol exposure, but a much smaller effect in male infants with prenatal alcohol exposure. In contrast, at 6.5 months miR-125a-5p was significantly elevated in plasma samples of male infants with prenatal alcohol exposure but not in plasma samples of female infants with prenatal alcohol exposure.

4. Fetal and adult brain tissues and blood serum

4.1. Mouse studies

Timed pregnancies of Swiss mice (RjOrl:SWISS) were from setting up 1 male and 2 females by Altounian et al.[65] Resulting pups were used at embryonic day E15.5 for *in utero* electroporation, postnatal days 0–8 for the analysis of corpus callosum axon growth and targeting, or at adult stage (> 8 weeks) for the analysis of long-term outcomes and behavior. Alcohol-naive pregnant mice were treated by daily intraperitoneal injection of 25 % ethanol in 0.9 % saline (final 2.0 g/kg) or saline (control) from E15.5 to E18.5. All animals were allocated to the treatment groups randomly. Regions corresponding to the corpus callosum and pre-motor/pre-sensory cortex were manually dissected from 150-mm thick vibratome sections of unfixed brains from control or alcohol-exposed individuals at postnatal day 0. MiRNA expression in cortex and microdissected corpus callosum regions of alcohol-exposed and control brains at postnatal day 0 was determined by RT-qPCR. The expression of miR-17–5p was significantly decreased following alcohol-exposure, and that of miR-9 (3 control and 3 prenatal alcohol exposure samples from 3 litters, 10 brains per sample).

In a study,[66] C57BL/6 J female mice had access to 10 % (w/v) ethanol in 0.066 % (w/v) saccharin or 0.066 % (w/v) saccharin solution for 4 h each day before mating to establish a consistent drinking pattern, and access was maintained throughout gestation. Alcohol consumption averaged (6.0 ± 0.5) g ethanol/kg/4 h, resulting in a blood ethanol concentration of (68.2 ± 9.1) mg/dl after 4 h. Brain cortices were isolated from E18 alcohol-exposed and controls (pooling all cortices from each dam) following removal of the meninges. RT-PCR was used to identify differential expression of miRNAs during development between E18 alcohol-exposed and saccharin cortices. MiRNAs were screened that had previously been found to be altered in the circulation of women who consumed alcohol during pregnancy.[57] Expression levels of miR-150–5p were significantly upregulated for both males and females in the alcohol-exposed group compared to the control group. The levels of other miRNAs, such as miR-191–5p, -340–5p, −342, were not significantly different between the two groups: angiogenic miRNAs such as miR-19a, -19b–1 were also unchanged. Interestingly, miR-126–3p, a miRNA abundant in endothelial cells, was upregulated in the alcohol-exposed cortex group compared to control group.

C57BL/6 J males 9-week-old were paired with a single nulliparous female 7–8 weeks-old overnight.[67] At 0.5 days post-coitum, the females were randomly assigned to either the ethanol-exposed group (10 % v/v ethanol) or control group (water). The volume of liquid consumed was measured every 24 h. Following 8 days of exposure, ethanol-exposed females were given water for the remainder of the study. Hippocampi were dissected from male ethanol-exposed and control offspring (n = 6/grp) at postnatal day 87, frozen, and stored at −80°C. Using miScript miRNA PCR Array, of 944 mature miRNAs assayed in the adult hippocampus (n = 6/grp), 488 were expressed in both the ethanol-exposed and control groups. Of these, 15 miRNAs were differentially expressed (all upregulated > 1.5-fold- change: miR-669a-5p, 467a*, 3097–3p, 135a, 467b–5p, 669b*, 135b, 487b, 450b–5p, 466c–5p, 592, 467e, -380–5p, 369–3p, 335–3p). 7 miRNAs were selected for TaqMan PCR validation. 4 miRNAs, miR-135a, 135b, 467b–5p, 487b–3p, were significantly upregulated in ethanol-exposed mice.

Mantha et al.[68] time-mated 8-week-old C57BL/6 J (B6) female mice with 8- to 12-week-old B6 males. Dams were injected s.c. with 2.5 g/kg ethanol in 0.15 M saline at 0 and 2 h on gestation days 14 and 16 (long-term group) or on gestational day 16 (short-term group).[69] This dosage could induce FASD-relevant neurodevelopmental and behavioral abnormalities. In adult mice, the first ethanol injection of 2.5 g/kg causes a resulting blood alcohol concentration of 250 mg/dl; the second dose given at 2 h increases the blood alcohol concentration to approximately 500 mg/dl. Given that mice metabolize ethanol five times faster than humans, the blood alcohol concentration would fall within an equivalency range of 200–300 mg/dl for humans. Matched control dams were injected with saline. Dams for the short-term group were sacrificed 4 h after injections. Dams from the long-term group were allowed to give birth. The pups were raised to postnatal day 70, which represents maturity, and were sacrificed. A total of 12 whole brains (n = 6 ethanol, n = 6 control) were dissected from fetuses at gestation day 16 (short-term). Another 12 whole brains (n = 6 ethanol, n = 6 control) were dissected from adult males at postnatal day 70 (long-term). The tissue was frozen and stored at −80°C. Total RNA was extracted from each brain. Equal concentrations of RNA from 3 male brains (from 3 separate mothers) were pooled for each treatment to reduce litter effects. Each treatment group had 2 biological replicates (n = 12 mice/group, total n = 24). By microarray analysis, the number of differentially expressed genes in the short- and long-term analyses was 48 and 68, respectively, with no overlap between the two groups. In the short-term group, 30 of the 48 genes identified (63 %) were upregulated. In the long-term group, only 27 of the 68 genes (40 %) were upregulated. For microarray miRNA analysis, only the RNAs from postnatal day 70 males were used. Equal amounts of RNAs from three

males were pooled, each for two biological replicates ($n = 4$ arrays). Twenty miRNAs were differentially expressed with expression ranging from 1.39-fold increase (miR-2145) to −1.90-fold decrease (miR-466c−3p) in ethanol-exposed vs. matched control mice (upregulated: miR-1942, 1952, 1964, 2145, 302c, 343, 369–5p; downregulated: miR-10b, 1194, 146b, 184, 208b, 335–5p, 342–5p, 449b, 455, 466b−3p, 466c−3p, 466e−3p, 684). The expression of 16 genes (8 short-term, 8 long-term) was subjected to confirmation by qRT-PCR. No genes from the short-term group were confirmed. For the long-term group: down-regulation of calcium/calmodulin-dependent protein kinase IG (*Camk1g*, *Ccdc6*), early growth response 3 (*Egr3*), heat shock 70 kDa protein 5 (glucose-related protein 78 kDa, *Hspa5*) and *Xbp1*. qRT-PCR results for homer homolog 1 (Drosophilia, *Homer1*) and heat shock protein 90 kDa alpha (cytosolic), class A member 1 (*Hsp90aa1*) were opposite to the microarray results. Thromboxane A2 receptor (*Tbxa2r*) was not statistically significant.

Kleiber et al.[70] mated female C57BL/6 J (B6) mice 8–10 weeks of age overnight with 8–12-weeks-old male C57BL/6 J mice. For short-term samples, at postnatal day 7 male offspring were given s.c. injections of 2.5 g/kg ethanol in 0.15 M saline at 0 h and again at 2 h (total dose 5 g/kg) or saline. This treatment has previously been shown to induce peak blood alcohol levels > 0.3 g/dl for 4–5 h and be sufficient to cause neuronal apoptosis and FASD-related behaviors. Pups remained separated from the dam until sacrificed 4 h following the initial injection. For long-term samples, mice were treated with two doses of 2.5 g/kg ethanol or saline given 2 h apart at both postnatal days 4 and 7. These mice were reared by their biological mothers until weaning with 2–4 same-gender littermates at postnatal days 21 and 22. Male mice were sacrificed at postnatal day 60. The short-term samples (4 h after injection) were used to compare acute gene expression changes to those changes that were detectable in the adult brain following ethanol exposure during synaptogenesis (postnatal days 4 and 7) reported by Kleiber et al.,[69] as well as to evaluate changes to miRNA expression in the adult brain following ethanol exposure. Whole brain tissue was isolated, frozen, and stored at −80°C. Total RNA was isolated. mRNA expression array hybridization was performed. Each array consisted of pooled RNA from three non-littermate males to reduce litter-specific noise. In total, males from six different litters were used, with one ethanol-treated and one control pup from each litter. Each ethanol-treated sample was matched with a saline-treated littermate in the control samples. Giving a high (5 g/kg over 2 h) dose of ethanol to neonatal mice on postnatal day 7 is to model a binge-like exposure during the third trimester neurodevelopmental equivalent. Ethanol exposure at postnatal day 7 caused an acute alteration of 315 gene transcripts in the brain 4 h following exposure with 138 (44 %) showing upregulation at 4 h after ethanol exposure. Most gene expression changes were subtle, with 78 % (245/315) of gene expression changes showing less than a 1.3-fold difference. Eleven transcripts that were altered acutely following ethanol exposure at postnatal day 7 remained altered into early adulthood without additional ethanol treatment. The miRNA array identified 33 different miRNA transcripts that were altered in the brains of adult male mice that had been exposed to alcohol during early neonatal development. Fold changes ranged from −4.17 (miR-704) to 2.44 (miR-721). Most of the miRNAs were downregulated with 73 % (24/33) showing a negative fold change compared to the saline-treated controls (downregulated: let-7b*, let-7g*, let-7i*, miR-10b*, 15b*, 590–5p, 223, 297a, 335–3p, 343, 34b−5p, 376b, 466i, 467a–1*, 503*, 544, 665, 1903, 1927, 1947, –-1970, 696, 704; upregulated: miR-184, −1941–5p, 26b, 467b, 669a, 878–3p, 93*, 1195, 721).

In a study by Stringer et al.,[71] a C57BL/6 J mouse model of binge-like exposure during the period of synaptogenesis was used, which was done by exposing mice to two acute doses of alcohol (5 g/kg) at neurodevelopmental stage equivalent to the third trimester of human pregnancy. This induces a peak blood alcohol level of > 3 g/L for 4–5 h following injection and is sufficient to cause neuronal apoptosis and FASD-related behaviors. Gene expression data were previously generated through microarray analysis (GEO # GSE34539) of RNA isolated from whole brain tissue of 60-day-old male mice exposed to binge-like levels of alcohol during the third trimester equivalent on postnatal days 4 and 7. MiRNA expression data (GEO # GSE 34413) was also generated from the same sample. Analysis of these data showed a reduction of *Canabinoid receptor 1* (*Cnr1)* (fold-change −1.33) in ethanol-treated brains compared to saline controls. Also, miR-26b was increased in ethanol-treated brains (fold-change 1.28) compared to saline controls. RT-qPCR confirmed the reduction of *Cnr1* transcript with a fold-change −1.14 in expression in ethanol-treated male brains compared to matched controls. Furthermore, the level of miR-26b was significantly increased (fold-change 3.71) in ethanol-treated male brains ($n = 5$) compared to matched controls ($n = 5$).

Laufer et al.[72] time-mated female C57BL/6 J (B6) mice ~8 weeks of age with male B6 mice. Pregnant dams were injected s.c. with two 2.5 g/kg doses of ethanol in saline (alcohol-treated) at 0 h and 2 h, or with saline (control), at gestation days 8 and 11 (neurodevelopmental trimester 1) or gestation days 14 and 16 (trimester 2).[73] The third trimester human equivalent occurs postnatally in mice,[74] and a binge exposure during this period was modeled by treating pups directly on postnatal days 4 and 7 via s.c. injection (trimester 3). Pups from one litter were matched across treatment groups for gender and weight to control for litter effects. Two doses of 2.5 g/kg of ethanol at 2 h apart were given, while matched controls received saline. All resulting offspring were weaned on postnatal day 21 and housed in cages with two to four same gender littermates. In alcohol exposure by voluntary consumption [continuous preference drinking (CPD)], pregnant females were placed in individual cages, given free access to 10 % ethanol and water for 2 weeks to establish a stable drinking pattern, then time-mated and provided both ethanol and water from gestation day 0 to postnatal day 10.[75] Control dams had access to water only. Voluntary maternal alcohol consumption was measured daily from gestation day 0 to postnatal day 10, and females drinking < 2 mL/d alcohol were excluded from the study. Resulting pups, both alcohol-exposed and matched controls, were weaned at postnatal day 21 and housed in cages of 2–4 same-gender mice. Alcohol-treated and matched control adult males (postnatal day 70) resulting from the four treatment paradigms ($n = 12$ per paradigm with 6 alcohol-exposed and 6 matched controls) were sacrificed. Whole brains were extracted, frozen, and stored at −80°C. Whole-brain total RNA was isolated. Equal amounts of total RNA from non-littermate males (three for the injection models and two for the CPD model) were pooled per biological replicate to reduce litter effects, with no litter contributing more than one individual. Two biological replicates (arrays) were used for each injection model, and three for the voluntary maternal drinking (CPD) model. MiRNA arrays showed that fetal alcohol exposure resulted in changes to global miRNA expression. Treatment during trimester 1, 2 or 3 resulted in the unique expression for 21/24 (88 %), 38/45 (84 %) and 60/68 (88 %) of the affected miRNAs for each of the three trimesters, respectively. The number for the voluntary consumption paradigm was also comparable [28/32 (88 %)]. In the maternal voluntary consumption mouse model, overall, 34 genes from the gene expression arrays showed inverse pairwise relationships with 1–13 miRNAs from the miRNA expression arrays that were predicted to target them. Of the 34 identified target genes, four (*Pten*, *Nmnat1*, *Slitrk2* and *Otx2*) are of particular interest in regard to FASD, owing to their roles in the brain. Upregulated miRNAs were: miR-152, 1224, 431, 743a, 17*, 200a*, 146b, 19b, 151, 679–5p; and downregulated: miR-369–5p, 25, 495.

In a study by Wang et al.[50] female C57BL/6 J mice (10 ± 1) weeks of age were mated with C57BL/6 J males, and pregnant females were randomly assigned to five groups receiving: water; maltose/dextrin (7.1 g/kg/d, 35.5 % w/v maltose/dextrin solution to provide the calorific equivalence of intermediate dose ethanol); low dose ethanol (2.0 g/kg/d, 12.67 % v/v ethanol solution); intermediate dose ethanol (4.0 g/kg/d, 25.34 % v/v ethanol solution); or high dose ethanol (6.0 g/

kg/d, 38.01 % v/v ethanol solution). Pregnant mice received one of the above solutions twice daily at 9 am and 11 am via gavage from gestation day 6 to gestation day 15. On gestation day 12, maternal tail blood samples were collected at 1 h after the second dose of ethanol. On gestation day 17, individual pregnant mice were euthanized followed by decapitation. The litter of each pregnant animal was delivered by cesarean section. Total RNA was extracted from fetal brains. The mean maternal blood ethanol concentrations in pregnant mice treated with 2.0, 4.0 or 6.0 g/kg/d ethanol were [0.29 ± 0.14], [1.25 ± 0.28] and [2.50 ± 0.24] mg/mL, respectively. Maternal death and spontaneous miscarriage increased with increasing ethanol dosage. There were three incidences of spontaneous miscarriage and one incidence of maternal death in the high ethanol dose group, and one incidence of spontaneous miscarriage and two incidences of maternal death in the intermediate dose group. By microarray analysis of fetal brains at gestation day 17.5, of the screened miRNAs, miR-10a, 10b, 9, 145, 30a–3p, -152 were upregulated, whereas miR-200a, 496, 296, 30e–5p, 362, 339. 29c, 154 were downregulated. MiR-10a and 10b showed the greatest expression in ethanol-exposed fetal mouse brain, with a fold change of 2.88 and 2.38, respectively, compared to control mice. RT-PCR confirmed the upregulation of miR-10a and −10b in fetal brains exposed to ethanol.

4.2. Rat studies

Gerace et al.[76] removed hippocampi from the brains of male and female Wistar rat pups at postnatal day 7. Transverse slices (420 μm) were prepared and transferred on to 30 mm diameter semi-porous membranes inserts (4 slices/insert) which were placed in 6 well tissue culture plates containing 1.2 mL medium/well. Typically, slices were maintained for 10 days to reach maturity *in vitro*. Organotypic hippocampal slices were used after 2 days *in vitro*. All the slices were screened for viability; any displaying signs of neurodegeneration were discarded from the study. Hippocampal slices were exposed for 7 days to 150 mM ethanol after 2 days (immature) culture *in vitro*. To reproduce a chronic exposure of ethanol, the medium was changed every day adding ethanol to the fresh culture medium (chronic ethanol). For control slices, the medium was changed every day by adding fresh culture medium. Following exposure to 150 mM (6.9 g/L) ethanol the concentration of ethanol in the medium was reduced by 15 % at 1 h later and by 79 % 24 h later, resulting in an average daily concentration in the range 127–32 mM (5.8–1.5 g/L). The maximal ethanol concentration detected in hippocampal slices was 2 mM (0.1 g/L) 1 h after ethanol exposure. Using RT-PCR, the levels of miR-137 and −501–3p were assessed after acute ethanol application (150 mM, 30 min) or chronic ethanol exposure (150 mM, 7 days). Immature hippocampal cultures exhibited increased levels of miR-137 and −501–3p after chronic but not after acute exposure to ethanol.

Male and female Sprague-Dawley rats were mated overnight by Balaraman et al.[77] On postnatal day 4, pups were randomly assigned to groups in a 2 (ethanol, sham) x 2 (choline, saline) x 2 (male, female) design. To minimize litter effects, no more than one gender pair per litter was assigned to a treatment group. A total of 48 animals (6 per group) was generated. Subjects received 2.625 g/kg ethanol via oral intubation in a nutritionally balanced milk formula (11.9 % v/v) twice per day, 2 h apart, for a daily dose of 5.25 g/kg/d from postnatal day 4 to postnatal day 9, equivalent to the third trimester of human pregnancy.[78] Ethanol-exposed subjects were also given two additional intubations of milk formula with no alcohol (2 h apart) to minimize growth differences. Sham controls received intubations, but no formula during the four daily intubations. From postnatal days 4–21, subjects were injected s.c. with choline chloride (100 mg/kg/d) or saline. On postnatal day 6, 20 μL of blood was collected via a tail clip, 1.5 h after the second ethanol treatment. On postnatal day 22, juvenile subjects were sacrificed, brains rapidly removed, the hippocampus was dissected and frozen, and total RNA extracted. No differences in body weight occurred among groups on postnatal day 4, but on subsequent days, ethanol-exposed subjects lagged in growth. All groups gained weight over days. During choline treatment, ethanol-exposed subjects continued to weigh less than controls. To assess the effects of choline on ethanol's effects specifically, those miRNAs were selected that exhibited an average of 1 cycle threshold CT (2-fold) difference between control and ethanol-exposed groups, i.e., those miRNAs that may be vulnerable to alcohol exposure. Of the 760 assessed miRNAs, 97 miRNAs fitted this criterion. Post-hoc ANOVAs showed that five miRNAs, miR-130b, −326, −374–5p, −878–3p, and −327 exceeded an unadjusted criterion of $P < 0.05$ for the main effects of group. Except for miR-878–3p, ethanol increased these miRNA levels and choline attenuated these effects. Post-hoc analyses showed that ethanol by itself significantly increased miR-200c and that choline treatment ameliorated ethanol's effects. Importantly, choline treatment ameliorated ethanol's effects, so that the miR-200c levels within the ethanol + choline group were not significantly different from that of controls ($P < 0.866$) but were significantly lower compared to the ethanol + saline group ($P < 0.005$). A pattern similar to that of miR-200c was found with miR-326 expression: ethanol increased its expression, while choline attenuated this effect. Analyses confirmed that miR-326 expression was significantly elevated in ethanol-exposed females, but not males, and that choline significantly ameliorated this effect. Ethanol also increased miR-374–5p expression above control levels and choline attenuated ethanol's effects to control levels. Likewise, ethanol also significantly increased expression of miR-130a and choline reduced its expression. Ethanol significantly increased the expression of miR-878–3p.

Ignacio et al.[79] received timed pregnant Long Evans rats on gestation day 4 (with gestation day 1 designated as the first day on which a sperm-positive plug was noted). On gestation day 12, dams received an initial i.p. injection of ethanol (2.9 g/kg as a 20 % v/v solution in saline) followed by a second i.p. injection 2 h later (of 1.45 g/kg ethanol). Control animals received i.p. injections of equivalent amounts of saline at the same time-points. After birth, all litters were culled to 10 pups within 24 h, with equal ratios of males/females as best as possible. On postnatal day 21, litters were weaned and male and female offspring were housed separately. After social behavior testing, animals were euthanized prior to decapitation, and brains rapidly removed, frozen, and stored at −80°C. The whole amygdala and ventral striatum were dissected from a total of 72 42-day-old male and female rats. A previous behavioral study on the same cohort of rats[80] examined the effects of a form of environmental manipulation termed social enrichment, during the post-weaning and early adolescent period (postnatal days 21–42) in animals prenatally exposed to ethanol. Prenatal ethanol exposure negatively affected social motivation performance in both male and female rats, but was completely reversed by social enrichment. The RNA-Seq analysis identified 1063 precursor and mature miRNAs. In the amygdala, of the 601 total miRNAs, 291 miRNAs were identified with consistent changes (in the same direction) due to ethanol in non-enriched animals compared to non-enriched controls representing 48 % directional concordance. Of these, 12 were changed in both RNA-Seq and Affymetrix microarray RNA GeneChips platforms (upregulated: miR-155, −34c; downregulated: miR-1843a-3p, 221–5p, 29c-3p, 384–5p, 412–3p, 129–1, 138–2, 322–2, 496, 9a–2). An additional 17 miRNAs only found by RNA-Seq were also observed to change significantly due to ethanol effects in non-enriched rats (upregulated: miR-15b-3p, 301b–3p, 448–3p, 449a–5p, 204, 3084a, 448; downregulated: miR-148a-5p, 221–3p, 222–3p, 299a–5p, 495, 6329, 667–3p, 299a, 3556b–2, 6329). In the ventral striatum, 281 of the miRNAs (47 %) showed concordant directional changes due to ethanol in non-enriched animals compared to non-enriched control animals, with 3 changed in both platforms (upregulated: let-7c-1, let–7c–2–3p, 542–1) and 11 additional miRNAs significantly changed that were only found by RNA-Seq (upregulated: miR-133b-3p, 345–3p, 6314, 6314; downregulated: miR-1247–5p, 489–5p, -493–3p, 540–5p, -122, 1306, 3591). Several miRNA changes in amygdala caused by ethanol were reversed by social enrichment, including miR-204, −299a, 384–5p, 222–3p, 301b–3p, and 6239.

Those miRNAs having altered expression in the human studies and the mouse and rat studies are summarized in Tables 1 and 2.

Table 1
Alterations of miRNA expression in maternal and infant blood and fetal brain tissue after alcohol consumption by pregnant women.

Study	Analysis method	Alcohol consuming *vs.* No alcohol; number, gender and mean age of mothers and their infants/fetuses		Altered miRNA expression in alcohol-exposed mothers and infants
Maternal blood plasma				
[59]	miRNome PCR panel	HEa 34 GA at enrolment (18.3 ± 5.1) wk HEua 23 GA enrolment (19.8 ± 5.1) wk	UE 36 GA at enrolment 18.0 ± 5.5) wk	Several maternal miRNAs were identified in both HEa and HEua groups, and while no effect of PAE (relative to the UE group) was observed in the composite sample, there was a significant effect of PAE when the samples were analyzed by gender: *Male specific-alcohol sensitive second trimester:* miR-361–5p, −22–3p, −328–3p, −146a−5p, −106b−5p, −25–3p, −let−7c−5p, −151a−3p, −320a, −223–5p *Male specific-alcohol sensitive third trimester:* miR-let−7f−5p, −30a−5p, −652–3p, −425–5p, −423–3p, −625, 3p, −532–3p, −148a−3p, −335–5p, -let−7c−5p, −126–5p, −125b−5p, −99a−5p *Female specific-alcohol sensitive second trimester:* miR-454–5p, −1972, −26b−5p, −106b−3p, −143–3p, −199a−5p, −150–5p, −339–5p, −1245a *Female specific-alcohol sensitive third trimester:* miR-329–3p, −320b, −2110, −125b−5p, −638
[60]		A literature review was conducted on miRNA levels in gestational pathologies caused by impaired placentation.		Placental and plasma levels of 8 of 11 HEa miRNAs were significantly dysregulated in one or more of these gestational pathologies with expression of the majority of these 8 miRNAs altered in both fetal growth restriction and preeclampsia. Eight of plasma HEa miRNAs in the second and third trimesters each significantly explained between 7 % and 19 % of infant variation in measures of neonatal height, weight, and head circumference (miR-299–3p, −491–3p, −885–3p, –518f−3p, −204–5p, −519a−3p, −222–5p, −449a).
[57]	RT-PCR	HEa 22 GA at delivery (37.9 ± 2.5) wk HEua 23 GA at delivery (40.0 ± 1.1) wk	UE 23 GA at delivery (40.0 ± 1.1) wk	*Upregulated:* miR-222–5p, −187–5p, −299–3p, −491–3p, −885–3p, –518f−3p, −760, −671–5p, −449a, −204–5p, −519a−3p at mid and late pregnancy
Maternal blood serum				
[56]	RT-PCR	40 alcohol-consuming subjects (GA 15.5 ± 1.3) wk	40 controls no alcohol GA (15.2 ± 1.4) wk	*Downregulated:* miR-124–3p, −132–3p, −134–5p, −138–5p, −302b−5p, −346, −9–5p in first trimester *Upregulated:* miR-124–3p, −125b−5p, −132–3p, −134–5p, −138–5p, −302b−5p, −346 in second trimester
[58]	RT-PCR	14 alcohol-consuming subjects GA at delivery (38.2 ± 2.2) wk	16 controls no alcohol GA at delivery (37.7 ± 3.7) wk	*Upregulated:* miR-509–5p, −4657, −542–3p *Downregulated:* miR−602 at end of pregnancy
Infant blood plasma				
[55]	qPCR	37 PAE group GA at delivery (38.9 ± 1.9) wk Infant gender 46 % male 6.5-mth WHO growth z-score Weight for age (−0.8 ± 1) Length for age (−1.2 ± 1) Head circumference for age (−0.9 ± 0.8)	31 controls GA at delivery (39.2 ± 1.9) wk Infant gender 46 % male 6.5-mth WHO growth z-score Weight for age (0.3 ± 0.8) Length for age (−0.4 ± 1.1) Head circumference for age (−0.1 ± 0.8)	At 2 wk *Upregulated:* miR-663b, −320d, −30c−5p, −21–5p, −143–3p, −22–3p, −1972, −624–5p, −18a−3p, −92a−3p, −598–3p, −23a−5p, −30e−5p At 6.5 mon *Upregulated:* miR-126–3p, −328–3p, −30c−5p, −24–3p, −193a−5p, −103a−3p, −92a−3p, −143–3p, −140–5p, −23b−3p, −125a−5p, −194–5p, −30b−5p, −148a−3p, −16–5p, −19b−3p, −423–5p, −101–3p, −27b−3p, −532–5p, −23a−3p, −223–3p, −125b−5p, −30a−5p
Fetal brain tissue				
[56]	RT-PCR	40 alcohol-consuming subjects GA (15.5 ± 1.3) wk	40 controls no alcohol GA (15.2 ± 1.4) wk	*Upregulated:* miR-124–3p, −302b−5p in the first trimester *Downregulated:* miR-125b−5p, −132–3p, −9–5p in the first trimester *Upregulated:* miR-125b−5p, −134–5p, −9–5p in the second trimester *Downregulated:* miR-124–3p in the second trimester

All the miRNAs listed are human miRNAs (hsa-miRs). GA: Gestational age; HEa: heavily alcohol-exposed mother with an FASD-affected child; HEau: moderate to heavily alcohol-exposed mother with an apparently unaffected child; mon: months; PAE: prenatal alcohol exposure; qPCR: quantitative polymerase chain reaction; RT-PCR: real time polymerase chain reaction; UE: low alcohol consuming or unexposed mothers with an unaffected child; wk: weeks.

Table 2
Alterations of miRNA expression in fetal, infant and adult brain tissues and blood serum in ethanol-exposed mice and rats.

Study	Analysis method	Alcohol treatment *vs.* no alcohol; number, gender and mean age of subjects		Altered miRNA expression in ethanol-exposed animals
Mouse studies				
[65]	RT-PCR	Swiss mice (RjOrl:SWISS) Maternal ethanol treatment E15.5-E18.5B rains PD0-PD8 10	Control E15.5-E18.5B rains PD0-PD8 10	Cerebral cortex and corpus callosum were dissected. For neonate mouse PD0 cortex *Downregulated:* miR-17–5p, −9–5p, −9–3p For neonate mouse PD0 corpus callosum *Downregulated:* miR-17–5p, −17–3p, −9–5p, −9–3p
[66]	RT-PCR	C57BL/6 J mice Maternal voluntary consumption of Ethanol GD0 to GD18B rains E18 7–8	Control access to saccharin GD0 to GD18B rains E18 7–8	For fetal E18 cerebral cortices *Upregulated:* miR-150–5p (in both males and females), −126–3p
[67]	PCR	C57BL/6 J mice Maternal voluntary consumption of Ethanol GD0 to GD8.5B rains PD87 6	Control access to water only GD0 to GD8.5 Brains PD87 6	In adult male mice, of 944 mature miRNAs assayed in the adult hippocampus, 488 were expressed in both the ethanol-exposed and control groups. Of these, 15 miRNAs were differentially expressed. 7 miRNAs were selected for TaqMan PCR validation In hippocampus *Upregulated:* miR-135a, −135b, −467b−5p, −487b−3p In blood serum *Upregulated:* miR-135a, −135b, −467b−5p
[68]	Mouse gene arrays miRNA array	C57BL/6 J mice Short-term GD16: Ethanol-exposed. Brains 6 Long-term PD70: Ethanol-exposed GD14 and GD16 Brains 6	Control Brains 6 Control Brains 6	The number of DEGs in the short- and long-term analyses were 48 and 68, respectively. There was no overlap among DEGs between the short- and long-term groups. Of the 48 genes identified in the short-term group, 30 (63 %) were upregulated. In the long-term group, only 27 of the 68 genes (40 %) were upregulated. For adult male mice PD70 *Upregulated:* miR-1942, −1952, −1964, −2145, −302c, −343, −369–5p *Downregulated:* miR-10b, −1194, −146b, −184, −208b, −335–5p, −342–5p, −449b, −455, −466b−3p, −466c−3p, −466e−3p, −684
[70]	mRNA expression array; hybridization miRNA array	C57BL/6 J mice Short-term PD7: 4 h following initial ethanol injection Brains 6 Long-term PD60: ethanol injections on PD4 and PD7 Brains 6	Control Brains 6 Control Brains 6	For male mice, ethanol exposure at PD7 resulted in the acute alteration of 315 gene transcripts in the brain 4 hr following exposure with 138 (44 %) showing upregulation 4 h after ethanol exposure (short-term exposure PD7) *Upregulated:* miR-184, −1941–5p, −26b, −467b, −669a, −878–3p, −93*, −1195, −721 *Downregulated:* let−7b*, let-7g*, let−7i*, miR-10b*, −15b*, −590–5p, −223, −297a, −335–3p, −343, −34b−5p, −376b, −466i, −467a−1*, −503*, −544, −665, −1903, −1927, −1947, −1953, −1970, −696, −704
[71]	Microarray analysis; RT-PCR	C57BL/6 J mice Long-term PD60: Ethanol injections on PD4 and PD7 Brains 5	Control Brains 5	For adult male mice, there was a reduction of *Cnr1* in ethanol-treated brains as compared to saline controls. *Upregulated:* miR-26b
[72]	miRNA arrays	C57BL/6 J mice Maternal voluntary consumption of ethanol GD0 to PD10 Brains PD70 6	Control access to water only Brains PD70 6	For adult male mice, in the maternal voluntary consumption mouse model, overall, 34 genes from the gene expression arrays showed inverse pairwise relationships with 1–13 miRNAs from the miRNA expression arrays that were predicted to target them. Of the 34 identified target genes, four (*Pten*, *Nmnat1*, *Slitrk2* and *Otx2*) are of interest in the context of FASD, owing to their roles in the brain. *Upregulated:* miR-152, −1224, −431, −743a, −17*, −200a*, −146b, −19b, −151, −679–5p *Downregulated:* miR-369–5p, −25, −495
[50]	Microarray analysis	C57BL/6 J mice Ethanol treatment by gavage 2x/d GD6 to GD15 Brains GD17.5	Control	Upregulated: miR-10a, −10b, −9, −145, −30a−3p, −152 Downregulated: miR-200a, −496, −296, −30e−5p, −362, −339. −29c, −154
Rat studies				
[76]	RT-PCR	Wistar rats PD7 Ethanol-exposed 150 mM 7 d Hippocampal slices 6 preparations	Control fresh medium Hippocampal slices 6 preparations	In hippocampal slices from rats PD7 and 2 d *in vitro* (immature) After chronic exposure to ethanol for 7 d *Upregulated:* miR-137 and −501–3p
[77]	TaqMan microRNA	Sprague−Dawley rats Ethanol-exposed PD4-PD9 Hippocampus PD22 6	Control no ethanol PD4-PD9 Hippocampus PD22 6	In adult rats PD22 *Upregulated:* miR-200c, −326 (only for females), −374–5p, −130a, −878–3p

(*continued on next page*)

Table 2 (*continued*)

Study	Analysis method	Alcohol treatment *vs.* no alcohol; number, gender and mean age of subjects		Altered miRNA expression in ethanol-exposed animals
[79]	RNA-Seq analysis	Long Evans rats GD4 Ethanol-exposed on GD12 ± social Enrichment PD21-PD42 Amygdala/ventral striatum PD42	Control no ethanol Amygdala/ventral striatum PD42	For adult rats PD42 In amygdala and no social enrichment *Upregulated:* miR-155, −34c, −15b−3p, −301b−3p, −448–3p, −449a−5p, −204, −3084a, −448 *Downregulated:* miR-1843a−3p, −221–5p, −29c−3p, −384–5p, −412–3p, −129–1, −138–2, −322–2, −496, −9a−2, −148a−5p, −221–3p, −222–3p, −299a−5p, −495, −6329, −667–3p, −299a, −3556b−2, −6329 In ventral striatum and no social enrichment *Upregulated:*miR-let−7c−1, let−7c−2–3p, −542–1, −133b−3p, −345–3p, −6314 *Downregulated*: miR-1247–5p, −489–5p, −493–3p, −540–5p, −122, −1306, −3591

All the miRNAs listed are mouse miRNAs (mmu-miRs) and rat miRNAs (rno-miRs). DEGs: Differentially expressed genes; E: embryonic day; GD: gestational day; h: hours; PCR: polymerase chain reaction; PD: postnatal day; RT-PCR: real time polymerase chain reaction.

5. Clinical translation

A search of the NIH Clinical Trials website (https://clinicaltrials.gov) revealed one recent study titled “Fetal Alcohol Spectrum Disorder: Clinical Description and Search for Epigenetic Biomarker (EPI-TSAF)” (NCT06471335). Blood samples and buccal smears were taken from 18 patients in the FASD group, while only buccal smears were collected from 18 patients in the control group. MicroRNAs were extracted from blood plasma and buccal swabs. The aim of the study is to identify a specific epigenetic signature for FASD to provide early diagnostic markers and determine the origin of the microRNAs identified. The patients were born in Réunion, France, and the study started in June 2024 and completion was November 2024. Study results have not yet been posted.

6. Limitations

Several important limitations were identified in the studies reviewed: (1) there were only six human studies found for the review; (2) the sizes of the alcohol-exposed and control (no alcohol) groups in the animal studies were quite small (usually 6–10) and none of the human or animal studies had performed a power-size calculation to determine what group sizes are needed to minimize the risk of a Type I or Type II error; (3) none of the studies had performed a receiver operating curve analysis to determine which miRNAs have potential diagnostic power in distinguishing between the alcohol-exposed and control groups; (4) while many of the animal studies had used combined samples from male and female pups, gender is important as several miRNAs were indicated as being gender-specific in the human studies[59]; (5) normalization of miRNA expression levels was not reported in several studies[57,68,71]; (6) there were very few interventions that were trialed to lessen the effects of alcohol-exposure on the developing mouse or rat fetus; however, choline treatment was effective when given postnatally to ethanol-exposed rats,[77] and supplementation with folic acid reduced the observed abnormalities of embryos compared to mice receiving ethanol only.[50]

7. Discussion

Diagnosing FASD poses significant challenges, as there is an absence of a singular medical test to confirm the condition. Consequently, medical professionals have to depend on a combination of physical features, developmental assessments, and a detailed history of prenatal alcohol exposure, which can be difficult to obtain accurately from mothers. This can lead to potential misdiagnosis, especially when symptoms overlap with other neurodevelopmental disorders such as attention deficit hyperactivity disorder or autism spectrum disorder. Symptoms of FASD can vary widely between individuals, even with similar levels of prenatal alcohol exposure. Also, while facial features such as a smooth philtrum, a thin vermillion border, or small palpebral fissures are characteristic of FASD, they may not be readily apparent especially in milder cases or as individuals age. Many symptoms of FASD such as learning difficulties, behavioral issues, and attention problems are also seen in other neurodevelopmental disorders, thus making diagnosis difficult.[81,82]

MiRNA biomarkers have been identified for many neurological diseases and disorders,[83] and are promising biomarkers and drug targets for neurological diseases.[84,85] The studies reviewed here had performed miRNA profiling in maternal and infant blood and fetal brain tissue after alcohol consumption by pregnant women (6 studies, Table 1), and in fetal and adult brain tissues and blood serum in ethanol-exposed mice and rats (11 studies, Table 2). For the human studies, there were 3 on miRNAs in maternal blood plasma, 2 in maternal blood serum, and 1 in infant blood plasma. Two of these studies had included second and third trimester plasma samples,[54,59] and two had examined first and second trimester serum samples[56] and end of pregnancy serum samples.[58] The study in infant blood plasma had analyzed samples collected at 2 weeks and 6.5 months postpartum.[55] For the mouse studies, two had used whole brains from adult mice that had received ethanol injections on postnatal days 4 and 7, which is the human third trimester neurodevelopment equivalent.[70,71] One study had used whole brains of adult mice which had been ethanol-exposed on gestation days 14 and 16, which is the human second trimester equivalent.[68] In addition, one study had examined whole brains of adult mice that had been ethanol-exposed through maternal voluntary consumption of ethanol from gestation day 0 to postnatal day 10.[72] There was one study that had collected the hippocampus of adult mice that had been ethanol-exposed through maternal voluntary consumption of ethanol from gestation day 0 to gestation day 8.5 and represents the human first trimester equivalent.[67] Also, there were two studies that had examined cerebral cortex (one had included corpus callosum) in the fetus or neonate that had been ethanol-exposed through maternal ethanol injections or voluntary consumption of ethanol on embryonic day 15.5–18.5 which would be equivalent to the human second trimester[65] and on gestation day 0–18 which would be equivalent to the human first + second trimester.[66] Another study had examined fetal brains exposed to ethanol on gestation days 6–15 which would be equivalent to the human first + second trimester.[50] For the rat studies, two studies had examined hippocampus or amygdala/ventral striatum of adult rats that had been ethanol exposed on postnatal days 4–9[77] or on gestation day 12,[79] respectively. The other study had utilized hippocampal slices in culture from rats at postnatal day 7 and exposed to ethanol for 7 days.[76]

In the human studies, miR-148a-3p and miR-125b-5p were identified as male specific-alcohol sensitive miRNAs in third trimester maternal blood plasma[59] and were upregulated in infant blood plasma at 6.5 months postnatally.[55] In addition, miR-125b-5p was upregulated in second trimester maternal blood serum.[56] It is noted that miR-30c-5p, −143–3p, −92a-3p were upregulated in infant blood plasma at both 2 weeks and 6.5 months postnatally.[55] Moreover, miR-124–3p, −132–3p, −134–5p, −138–5p, −302b-5p, −346, −9–5p were downregulated in the first trimester and upregulated in the second trimester (not for miR-9–5p) in maternal blood serum.[56] The profile for miRNAs in fetal brain tissue would indicate that miR-125b-5p and −9–5p were downregulated in the first trimester and upregulated in the second trimester, and that miR-124–3p was upregulated in the first trimester and downregulated in the second trimester.[56] The direction of alterations in miR-125b-5p expression in the second trimester and of miR-9–5p in the first trimester were the same for maternal blood serum and fetal brain tissue.[56] The alterations in the eight miRNAs in maternal blood serum in the first and second trimester (miR-124–3p, −125b-5p, −132–3p, −134–5p, −138–5p, −302b-5p, −346, −9–5p) brought about by maternal alcohol exposure could possibly serve as potential biomarkers. This would be especially important as the first trimester is when women who have consumed alcohol become aware that they are pregnant, and interventions could be performed to lessen the adverse effects of alcohol on the developing embryo/fetus.

In the animal studies, for cerebral cortex and corpus callosum of neonates on postnatal day 0 from mated Swiss female mice (RjOrl:SWISS) receiving ethanol treatment on embryonic days 15.5–18.5, there was a downregulation of miR-17–5p, −9–5p, −9–3p.[65] In adult C57BL/6 J mice that had been exposed by maternal voluntary consumption of ethanol from gestation days 0–8.5, in brains removed on postnatal day 87 there was an upregulation of miR-135a, −135b, −467b−5p, −487b−3p in hippocampus and of miR-135a, −135b, −467b−5p in blood serum.[67] Also, miR-467b was upregulated in brains of C57BL/6 J male infants at 4 h after initial ethanol injections on postnatal day 7.[70] In addition, miR-26b and miR-878–3p were upregulated in brains on postnatal day 7,[70] while miR-26b was upregulated on postnatal day 60 of C57BL/6 mouse offspring having received ethanol injections on postnatal days 4 and 7[71] and miR-878–3p was upregulated in hippocampus of Sprague Dawley rats on postnatal day 22 having been ethanol-exposed on postnatal days 4–9.[77] At postnatal day 70, in the brains of male infants of C57BL/6 J mice, voluntary maternal consumption of ethanol on gestation day 0 to postnatal day 10 resulted in a downregulation of miR-495.[72] Also, a downregulation of miR-495 occurred on postnatal day 42 in the amygdala with no social enrichment of offspring of Long Evans rats that were ethanol-exposed on gestation day 12.[79] In fetal brains of C57BL/6 J mice exposed to ethanol by maternal gavage twice daily on gestation days 6–15, there was an upregulation of miR-9 and −152 and a downregulation of miR-496 and −29c on gestation day 17.5.[50] Also, on postnatal day 70 in brains of male infants of C57BL/6 J mice exposed by voluntary maternal consumption of ethanol on gestation day 0 to postnatal day 10, there was an upregulation of miR-152.[72] Downregulation of miR-496 and −29c−3p expression was observed in the amygdala on postnatal day 42 in rats with no social enrichment exposed to ethanol on gestation day 12.[79]

While some similarities were identified in the findings of miRNA expression in the mouse and rat studies (as described above), it is of interest to see if there are any similarities in miRNA profiles in the human and animal studies. However, it is only possible to compare data sets for miRNAs in fetal brain tissue as only one animal study measured miRNAs in blood. In the mouse and rat studies, attention was drawn to miR-17–5p, −9–5p, −9–3p,[65] −135a, −135b, −467b-5p, −487b-3p,[67] −26b, −467b, −878–3p,[70,71] −495,[72,79] −9, −152, −496, −29c.[50,72] In the human fetal brain tissue, miR-9–5p was downregulated in the first trimester and upregulated in the second trimester.[56] In the mouse, miR-9 was upregulated in fetal brain tissue on gestation day 17.5 having been exposed by maternal ethanol treatment on gestation days 6–15 which represents the human first + second trimester equivalent.[50] In neonate mice at postnatal day 0, miR-9–5p was downregulated in cerebral cortex and corpus callosum after maternal ethanol treatment on embryonic days 15.5–18.5, which represents the human second trimester equivalent[65] and is in the opposite direction to the human study. This could be due to having chosen a specific region of the brain for analysis. None of the other miRNAs that were indicated as possibly being important in fetal brain tissue from the animal studies were changed in the human studies.

In summary, some progress has been made in identifying possible miRNAs as biomarkers of pregnant women and their offspring who have been exposed to alcohol. Comparison between the results of the human and animal studies is limited by the small number of human studies and that the animal studies were performed with fetal and infant brain tissue. Additional human studies are needed to find potential biomarkers in blood samples of women who have heavily consumed alcohol during pregnancy and in blood samples of their infants. Also, in the animal studies it would be helpful to assay miRNAs in blood samples of pregnant dams and their offspring. Prenatal alcohol exposure was shown to reduce absorption and/or metabolism of folate and choline and to produce similar outcomes to maternal choline/folate deficiency.[86] Folate or choline deficiency can cause reduced blastocyst development and implantation, reduced placental invasion, vascularization and nutrient transport capability, impaired fetal brain development, and abnormal neurodevelopmental outcomes. Several studies have demonstrated that the effects of prenatal alcohol exposure on brain development can be alleviated by folate or choline supplementation.[86] Choline can reduce the severity of alcohol effects on the fetus after the alcohol exposure has occurred. When administered during the early postnatal period, choline targets alcohol's effects on behaviors associated predominately with hippocampal function.[34] Therefore, it would seem advantageous to treat pregnant women and their infants with folate and choline in cases where women indicate they have heavily consumed alcohol during pregnancy and the infants have been exposed to alcohol. Elevit DHA & choline (Bayer Australia Limited, NSW, Australia) is a formulation designed to support a healthy placenta during pregnancy as well as the development of a healthy central nervous system in the fetus. Also, the Elevit Pre-conception & Pregnancy multivitamin formulation contains folic acid. Maternal choline supplementation with 480 mg/d (approximates to an adequate intake of choline) or 930 mg/d during the third trimester showed that children at the age of 7 years in the 930 mg/d group had improved sustained attention (*vs.* 480 mg/d group) using a signal detection task.[87] Other tests administered to these children at the age of 7 years indicated that higher maternal choline intake during the third trimester also improved working memory and problem solving,[87] which is positively associated with school performance. Three recent human studies found that either maternal[88,89] or early postnatal[90] choline supplementation improved cognitive outcomes in children exposed to alcohol prenatally. The animal studies have shown that choline can alleviate the effects of alcohol and alter the levels of specific miRNAs in the fetal brain.[77]

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Declaration of Competing Interest

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