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Identifying Prenatal Alcohol Exposure and Children Affected by It: A Review of Biomarkers and Screening Tools

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PURPOSE: Early identification of prenatal alcohol exposure (PAE) and of those in need of services resulting from this exposure is an important public health concern. This study reviewed the existing literature on potential biomarkers and screening tools of PAE and its impact.
SEARCH METHODS: Electronic databases were searched for articles published between January 1, 1996, and November 30, 2021, using the following search terms: ("fetal alcohol" or "prenatal alcohol" or "FASD" or "alcohol-related neurodevelopmental disorder" or "ARND" or "ND-PAE") and ("screening" or "identification" or "biomarker"). Duplicate articles were electronically eliminated. Titles and abstracts were reviewed for appropriateness, and selected articles were retrieved for further analysis. Additional articles were added that were referenced in the reviewed articles or identified from expert knowledge. Information about the characteristics of the sample, the biomarker or screening tool, and the predictive validity outcome data were abstracted. A narrative analysis of the studies was then performed on the data.
SEARCH RESULTS: A total of 3,813 articles were initially identified, and 1,215 were removed as duplicates. Of the remaining articles, 182 were identified as being within the scope of the review based on title and abstract inspection, and 181 articles were successfully retrieved. Of these, additional articles were removed because they were preclinical (3), were descriptive only (13), included only self-report of PAE (42), included only mean group comparison (17), were additional duplicates (2), focused on cost analysis (9), missed predictive validity data (24), or for other reasons (23). The remaining articles (n = 48) were abstracted. An additional 13 manuscripts were identified from these articles, and two more from expert knowledge. A total of 63 articles contributed to the review.
DISCUSSION AND CONCLUSIONS: Biomarkers and screening tools of PAE and its impact fall short of ideal predictive validity characteristics. Higher specificity than sensitivity was found for many of the biomarkers and screening tools used to identify PAE and its impact, suggesting that current methods continue to under-identify the full range of individuals impacted by PAE. Exceptions to this were found in recent investigations using microRNAs related to growth and vascular development, proteomic changes associated with PAE, and combinations of markers estimating levels of various cytokines. Replications of these findings are needed across other samples to confirm the limited data available. Future research on biomarkers and screening tools should attend to feasibility and scalability of implementation. This article also recommends a systematic process of evaluation to improve early identification of individuals impacted by PAE so that harm reduction and habilitative care efforts can be implemented.

KEYWORDS: alcohol; prenatal alcohol; FASD; identification; biomarkers; fetal alcohol spectrum disorders; prenatal exposure delayed effects

Although the awareness of the negative impact of prenatal alcohol exposure (PAE) was already alluded to in ancient writings[1] and the impact of ethanol embryopathy in animal models was studied as early as 1910,[2] the conceptualization of a syndrome associated with PAE was not recognized within modern medicine until the mid-20th century.[3,4] The syndrome or disorder was not uniformly accepted, however, and debates occurred within the field related to the operationalization of criteria for making a clinical diagnosis. In 1996, a group of scientists were brought together under the auspices of the Institute of Medicine (IOM) to delineate criteria for a diagnosis and a public health care plan for addressing the needs associated with the condition.[5] This committee established the first consensus criteria for fetal alcohol syndrome (FAS) and recognized associated conditions, such as partial FAS (pFAS), alcohol-related birth defects (ARBD), and alcohol-related neurodevelopmental disorder (ARND). Various operational definitions of the IOM report's diagnostic guidelines have been used to make a clinical diagnosis.[6-17] In all cases, these diagnostic formulations struggle with identifying infants negatively impacted by PAE because few tools are available for assessing early brain development. In addition, many of the diagnostic formulations require input from complex medical teams evaluating different domains of impact, which are costly and heavily constrained by the number of professionals qualified to carry out the assessments.

Estimates of the prevalence of prenatal alcohol-related disorders have varied dramatically over the years. In the initial IOM report, which reviewed several registries and clinic-based studies, the estimate of FAS was reported to be in the range of 0.5 to 3 cases per 1,000 births;[5] however, more recent estimates have been much higher. A large consortium that estimated the prevalence of fetal alcohol spectrum disorders (FASD)—an umbrella term used to refer to a range of conditions (FAS, pFAS, ARBD, and ARND) associated with PAE—in four communities within the United States using active case ascertainment yielded a conservative estimate of 11.3 to 50 per 1,000 births[18] and an even higher weighted prevalence estimate of 31 to 99 per 1,000 births. A review of more than 24 unique studies carried out throughout the world resulted in a prevalence estimate of 8 per 1,000 births with a 95% confidence interval of 5 to 12 per 1,000 births.[19] Variations in the estimates are likely related to differences in diagnostic criteria used to estimate the prevalence of the disorder across studies, use of active versus passive surveillance methods, and regional variations in drinking patterns. Historically, documentation of PAE has been difficult to obtain due to unreliability of the self-report of women drinking in pregnancy and potential social stigma associated with acknowledging alcohol use in pregnancy that can result in underreporting of PAE.[20] The lack of recognition by various health professionals for the cluster of symptoms associated with the diagnosis of FASD also has contributed to under-recognition of those impacted by PAE.[21]

In anticipation of this problem, the IOM report outlined the need for biological markers of alcohol teratogenesis to help with resolving variations in case definitions.[5] The term "biomarker" refers to a broad collection of medical signs that can be used to identify a disease and can be measured accurately and reliably.[22] Biomarkers differ from medical symptoms, which are collected via patient report of their status and typically refer to biological measurements associated with the disease state. Biomarkers have the advantage of reducing ambiguity in patient reporting of symptoms but are only useful if they can validly predict a clinical endpoint—that is, if they can appropriately identify the disease state and avoid misclassification of individuals who do not have the condition. In the case of PAE, the clinical endpoint may be the identification of an alcohol-exposed pregnancy or of those negatively impacted by their exposure. Ideally, the identification would occur as early as possible during or after pregnancy to enhance opportunities for intervention. Identification during pregnancy could lead to harm reduction efforts, whereas early postnatal recognition of infants negatively impacted by PAE would increase the opportunities for access to habilitative care to optimize early brain development during phases of high neuroplasticity.[23] In addition to biomarkers, screening tools that sample symptoms of the disease state, or some combination of these, may be useful in identifying those negatively impacted by PAE. The development of innovative methods and tools that can be used to reduce the costly diagnostic assessment burden that constrains the identification of individuals in need of services are of particular value as such tools would allow for improved scalability and implementation in resource-poor areas of the world.

This review attempts to clarify potential advancements in the identification of biomarkers of PAE or its impact that could be used to improve early recognition of those adversely affected since the original IOM report's call for the development of biomarkers of alcohol-related teratogenesis. To this end, the authors conducted a review of the literature on the predictive validity of biomarkers or screening tools for identification of PAE or FASD and performed a narrative analysis of the findings.

Search Methods

Studies were considered for review if the article was published or available online between January 1, 1996, the first day of the IOM report publication year, and November 30, 2021. The target population consisted of individuals of any age who had been diagnosed with PAE or with a clinical disorder associated with PAE (i.e., FAS, pFAS, ARND, ARBD, and neurobehavioral disorder associated with prenatal alcohol exposure [ND-PAE]).[24]

In addition, the article's focus had to include screening or identification of PAE or one of the clinical disorders associated with PAE. The article also had to include empirical data related

to the screening or identification procedures and provide some aspect of the biomarker's predictive characteristics. Predictive validity characteristics evaluated in each study included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and area under the curve (AUC). Sensitivity refers to the probability that the test is positive when the condition is present. Specificity refers to the probability that the test is negative when the condition is not present. PPV refers to the probability that the condition is present when the test is positive. NPV refers to the probability that the condition is not present when the test is negative. Accuracy refers to the overall probability that the case is correctly classified from the test. Criterion descriptors for the predictive values are as follows: 90–100%, Excellent; 80–89%, Good; 70–79%, Fair; and below 70%, Poor. Finally, AUC is derived from creating receiver operating curves by plotting the true positive rate (sensitivity) relative to the false positive rate (1-specificity). The AUC references the area on the graph created by the regression line relative to the chance rate of prediction. Values of 1 would indicate perfect condition, and values of 0.50 would indicate chance prediction using a binary (yes/no) model.

Definitions for the first five predictive validity characteristics and formulas for computing them are outlined in Figure 1, a confusion matrix that illustrates the classic prediction modeling used when comparing a test's ability to identify a given state or condition. The confusion matrix is a contingency table that presents the frequency of individuals categorized across two dimensions, the actual true state of whether or not an individual has a disease or condition, and the predicted state derived from the results of the testing indicating the presence of the disease or not.

To identify studies, the following electronic databases were searched: PsycInfo, PubMed, Medline, Web of Science, ERIC, and the Cochrane Central Register of Control Trials. Search terms used were ["fetal alcohol" or "prenatal alcohol" or "FASD" or "alcohol-related neurodevelopmental disorder" or "ARND" or "ND-PAE"] and ["screening" or "identification" or "biomarker"]. Document type was limited to "articles," but no language restrictions were placed on the initial search. Despite extensive work in animal models of PAE on various promising biomarkers, only articles using humans were selected as the focus of this study was to analyze the current knowledge of potential tools that could be used to identify people affected by PAE. Preclinical biomarker methodologies still need translation into human populations to effectively evaluate their predictive characteristics.

References were then merged into Endnote X9.3.1 and screened for duplicates. The remaining studies were then reviewed to eliminate nonempirical studies (i.e., reviews or editorial articles) and those involving training of professionals to screen. Articles were also excluded if they established group differences without analyzing the predictive validity of the outcome or were descriptive of PAE in a given population. While establishing group differences may be a first step in establishing the utility of a biomarker or screening tool, such differences do not establish a tool's predictive utility. IQ tests are a classic example of tools that consistently demonstrate group differences between PAE groups relative to community samples without exposure;[25] however, they have little predictive utility when used independently as a result of the wide range of outcomes seen in

		True State: Positive	True State: Negative	
Test	Positive	True Positive A	False Positive C	PPV (Sensitivity x Prevalence) / (Sensitivity x Prevalence + (1 - Specificity) x (1 - Prevalence))
Test	Negative	False Negative B	True Negative D	NPV (Specificity x (1 - Prevalence)) / (1 - Sensitivity) x Prevalence + Specificity x (1 - Prevalence)
		Sensitivity A / (A + B)	Specificity D / (C + D)	Accuracy Sensitivity x Prevalence + Specificity x (1 - Prevalence)

Figure 1. Confusion matrix. The confusion matrix provides definitions of the various predictive validity terms within a contingency table where cases are plotted relative to the prediction variable and the designated "true state." True state refers to whether the individual has a disease or condition (positive) or does not have a disease or condition (negative), and the test reflects the outcome of the criterion used to indicate a positive or negative prediction of disease state. Sensitivity refers to the probability that the test is positive when the condition is present. Specificity refers to the probability that the test is negative when the condition is not present. PPV refers to the probability that the condition is present when the test is positive. NPV refers to the probability that the condition is not present when the test is negative. Accuracy refers to the overall probability that the case is correctly classified from the test. *Note:* NPV, negative predictive value; PPV, positive predictive value.

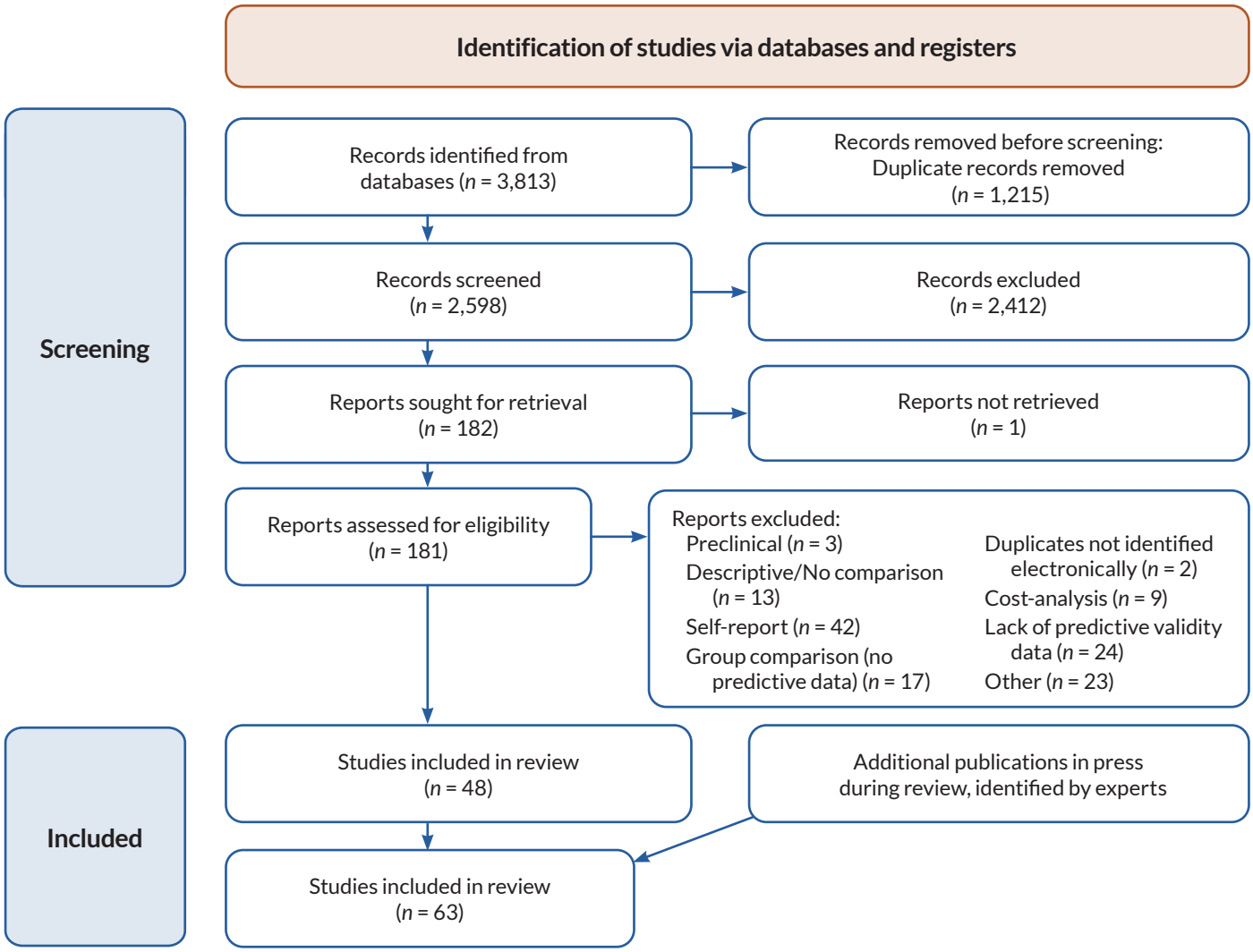

Figure 2. Flow diagram of the steps in the screening process for this review.

individuals with PAE and its associated overlap with comparison samples. A flow diagram (Figure 2) outlines the various steps in screening the articles and the number of articles at each step.

Search Results

A total of 3,813 articles were initially captured by the search, and 1,215 were identified as duplicates. Article titles and abstracts were then screened for inclusion, and an additional 2,412 were eliminated, leaving 181 full articles that were retrieved. One article could not be retrieved. The full articles were reviewed for appropriateness, and 133 articles were excluded for the following reasons: three were preclinical, 13 were descriptive only, 42 related to predictive utility of self-report methods of PAE, 17 were identified as group comparison studies, two were additional duplicates not identified electronically, nine were related to cost analysis, and 24 after further review did not have predictive data. This left 48 articles; however, upon further review, 13 additional articles were identified that were not retrieved by the search. Moreover, two additional articles were identified based on expert knowledge. This resulted in 63 articles included in the review.

Biomarkers and screening tools were categorized as predicting prenatal exposure status or alcohol-related teratogenesis in the offspring. Appendix 1 provides details on the articles that involved biomarker predictors of PAE status, and Appendix 2 provides details on biomarker predictors of FASD and associated symptoms. Both appendices list the articles in alphabetical order by the first author's last name as many involve the evaluation of several biomarkers and predictors within one study. Appendix 3 provides details on other screening tool predictors of FASD and associated symptoms, including craniofacial features, neurophysiological responses, neuroimaging analyses, questionnaire responses, and various test batteries assessing performance. As typically only one screening tool was evaluated within a study, Appendix 3 groups studies by screening tool category and then lists studies alphabetically.

Predictive validity information was obtained from information explicitly stated in the text or tables or was computed from information regarding cell sizes in the predictive validity tables provided in the article or as described in the text. Computations were performed using MedCalc software for diagnostic test evaluation (MedCalc Software Ltd, Ostend, Belgium). Predictive validity values are presented as percentages with the exception of AUC values, which were reported in proportions of accurate diagnostic classification with values of 0 to 1.00.

The sensitivity, specificity, accuracy, and AUC values were plotted on radial curves for each type of biomarker, with each type of predictive characteristic color-coded (see Figure 3). AUC values were multiplied by 100 to facilitate plotting them on the same curves as the other predictive values. The obtained values for each of the validity characteristics were provided for each unique outcome of the study. For studies that compared the biomarker response to common outcomes defined differently (e.g., self-report using different assessment tools), only the obtained values reflecting the least and greatest value were included to reflect the range of validity. Radial curves plot individual values of these predictive parameters along a curve with increasing number of indicators smoothing out until the curve is circular. The strength of the prediction is reflected along the radius of the circle so that values in the outer region reflect increased predictive validity and those in the inner region reflect lower levels of predictive validity. Radial curves allow for a quick visual analysis of each of the predictive characteristics for each type of biomarker or screening tool and the variation across the findings. Curves with more points along the outer ring with less deviance inward reflect increased predictive status and uniformity in the prediction.

Biomarkers

Biomarkers of PAE were derived from various biological samples obtained from mothers, including blood (plasma and dried blood spots), urine, hair, and fingernail clippings. Sources of biomarkers evaluated in the infant included blood (plasma and dried blood spots) and meconium. Additional biomarkers of PAE or its effects were obtained from placental tissue and the umbilical cord. Biomarkers were evaluated against group status determined from maternal self-report of alcohol consumption and the offspring's FASD symptomatology or diagnosis.

One group of biomarkers evaluated included fatty acid ethyl esters (FAEE) derived from hair or meconium. FAEE are metabolites of ethanol and provide a long-term estimate of alcohol consumption over the course of a pregnancy. They were analyzed either in a collective grouping of FAEE or individually (i.e., ethyl stearate, ethyl linoleate); in total, 30 obtained values or point estimates of predictive validity were provided across 12 studies.[26-37] In three additional studies, FAEE were used as the outcome to assess other biomarker predictors.[38-40] The radial graph of the predictive characteristics of FAEE in combination or separately (see Figure 3A) suggests that their specificity (range, 43%–100%; median, 83%) as biomarkers is significantly better than their sensitivity (range, 4%–100%; median, 65%); overall accuracy estimates fell in the poor to fair range (range, 62%–79%; median, 68%). Estimates of the AUC values were variable, ranging from poor to excellent (range, 0.52–0.93; median, 0.71). There was no clear pattern that a summation of several FAEE or any one FAEE provided better prediction.

Other biomarkers assessed included gamma-glutamyltransferase (GGT),[35,41-46] carbohydrate-deficient transferrin (CDT),[38,41-46] ethyl glucuronide (EtG),[30,31,35,38-41,43,44,47-52] ethyl sulfate (EtS),[31,35,41] and mean corpuscular volume (MCV).[45] GGT, CDT, and MCV provide an indirect assessment of the impact of heavy and chronic alcohol use on the mother's metabolic functioning. Estimates of GGT can be obtained from plasma, urine, and hair, whereas CDT and MCV estimates are only obtained from plasma. EtG and EtS are metabolites of ethanol that are present in hair, meconium, urine, and nails. Predictive validity information was found for seven studies using GGT (10 point estimates), seven studies using CDT (13 point estimates), and one study using MCV (three point estimates). Fifteen studies with 24 point estimates were identified for EtG. Three studies evaluated EtS,[31,35,41] but only two provide estimates of EtS alone,[35,41] whereas one study evaluated EtS in combination with EtG.[31] Consistently, these biomarkers provided fair to excellent specificity—EtG (range, 71%–100%; median, 87%); EtS (range, 97%–100%; median, 98%); CDT (range, 71%–100%; median, 95%); GGT (range, 71%–100%; median, 95%); and MCV (both values 100)—but exceptionally poor sensitivity—EtG (range, 0%–97%; median, 23%); EtS (range, 7%–15%; median, 7%); CDT (range, 5%–40%; median, 13%); GGT (range, 11%–50%; median, 25%); and MCV (values of 15 and 20).

One study evaluated postnatal serum levels of insulin-like growth factor-II (IGF-II) as predictors of FASD status in children or youth who either had a history of meconium FAEE levels above 2 nmol/g or had been adopted from Eastern European countries with confirmed PAE (two point estimates).[27] The participants were assessed for IGF-II levels below the 5th percentile. IGF-II levels below the 5th percentile had excellent specificity (99% and 100%, respectively) for predicting FASD status, but very poor sensitivity (13% and 39%, respectively) and overall accuracy (24% and 47%, respectively).

One study provided limited information on aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are both markers of impaired liver functioning, as biomarkers of PAE.[46] Only AUC values were provided, and these were poor (0.47 and 0.54, respectively).

Phosphatidylethanol (PEth) is a more recent biomarker of ethanol metabolism that has been evaluated in maternal and infant plasma and dried blood spots.[35,41,52-55] Six different studies found considerable variability in the predictive characteristics of PEth depending on the source of the PEth. Assays of maternal blood as well as plasma from the umbilical cord yielded a wide

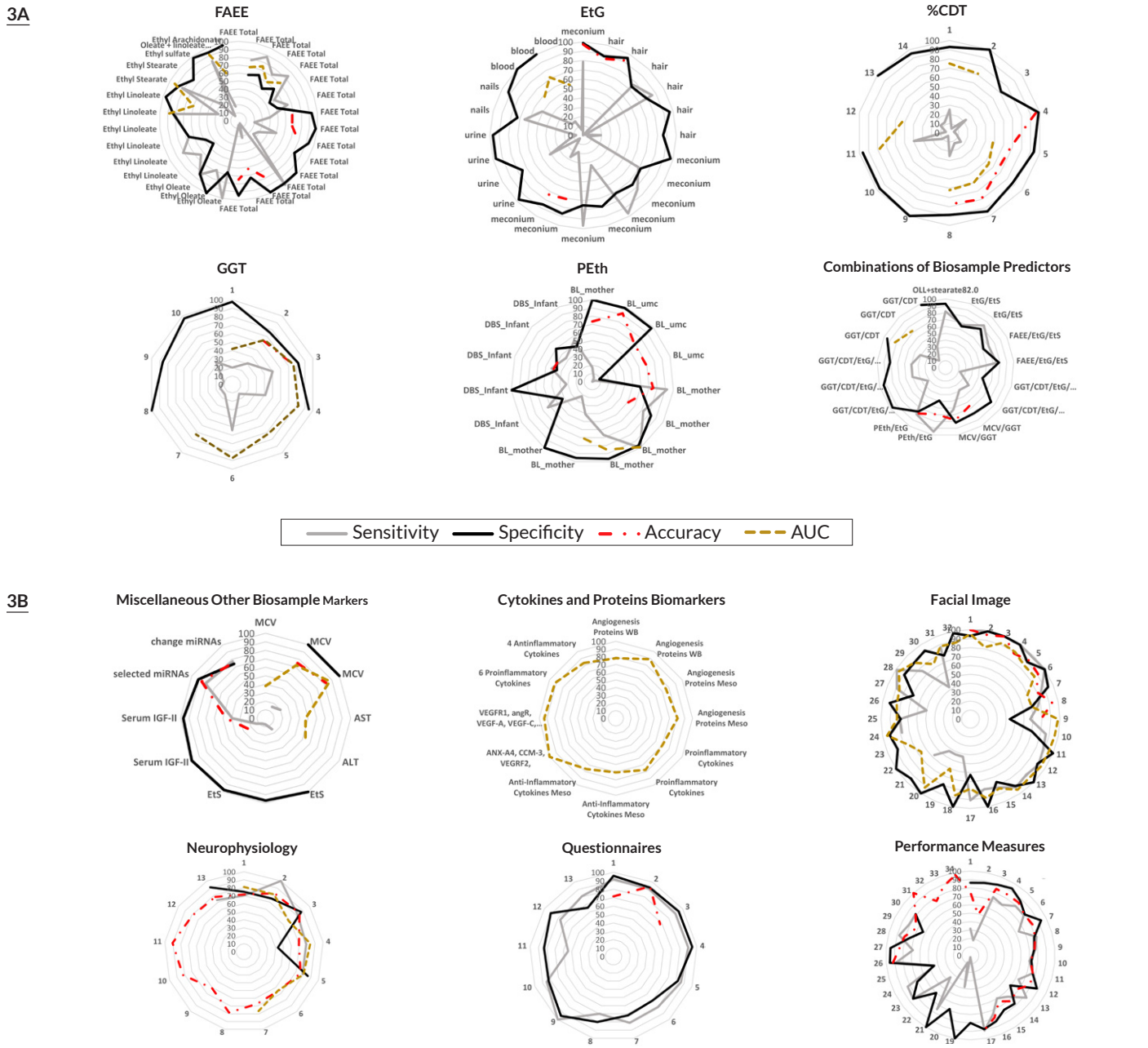

Figure 3. Radial curves of PAE biomarkers (A) and of biomarkers and screening tools for PAE and its impact (B). Radial graphs indicate the specificity (gray curves), sensitivity (black curves), accuracy (red dotted curves), and area under the curve (AUC) values (gold curves) relative to the criterion evaluated in the study. Point estimates or the obtained values of the validity characteristics were provided for each unique outcome of the study. For studies that compared the biomarkers' response to common outcomes defined differently (e.g., self-report using different assessment tools) only the point estimates reflecting the least and greatest value were included to reflect the range of validity. AUC values were multiplied by 100 to facilitate plotting them on the same curves as the other predictive values. The radial graph plots the various findings along curves with increasing prediction (0–100). Radial curves allow for a quick visual analysis of each of the predictive characteristics for each type of biomarker or screening tool and the variation across the findings. Greater numbers of findings displayed in a graph result in smoothing of the curve. The strength of the prediction is reflected along the radius of the circle so that values in the outer region reflect greater predictive validity and those in the inner region reflect lower levels of predictive validity. Curves with more points along the outer ring with less deviance inward reflect increased predictive status and uniformity in the prediction. Separate colored lines are used to connect the points along with curve for each of the predictive characteristics. Criterion descriptors for the values plotted above are as follows: 90–100, Excellent; 80–89, Good; 70–79, Fair; and below 70, Poor.

Note: ALT, alanine aminotransferase; angR, angR protein; ANX-A4, annexin-A4; AST, aspartate aminotransferase; AUC, area under the curve; BL, blood level; CCM-3, cerebral cavernous malformation 3 (a protein); CDT, carbohydrate-deficient transferrin; a protein; DBS, dried blood spots; EtG, ethyl glucuronide; EtS, ethyl sulfate; FAEE, fatty acid ethyl esters; GGT, gamma-glutamyltransferase; IGF-II, insulin-like growth factor-II; MCV, mean corpuscular volume; miRNAs, micro RNAs; NPV, negative predictive value; OLL, oleate + linoleate + linolenate; PEth, phosphatidylethanol; PPV, positive predictive value; umc, umbilical cord; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WB, Western Blotting Procedures.

range of specificity (range, 9%–100%; median, 96%), sensitivity (range, 0%–100%; median, 22%), and overall accuracy (range, 51%–91%; median, 71%). Tests of dried blood spots taken from infants also had variability in their predictive characteristics but were generally not as good as maternal blood and plasma obtained from the umbilical cord—specificity (range, 42%–100%; median, 95%); sensitivity (range, 32%–63%; median, 52%); and overall accuracy (range, 48%–50%; median, 50%).

Collectively, these results regarding the validity of biomarkers for predicting PAE status suggest that a positive response was not very effective in identifying the full range of individuals who self-reported prenatal alcohol use and missed many affected individuals. This was also true of the studies evaluating the predictive modeling of the impact of PAE (see Appendix 2). Combining biomarkers did not result in substantial improvements in the predictive characteristics (see Figure 3A, bottom right panel). As has been observed in other biomarker analyses, there appeared to be a trade-off such that as sensitivity of combined biomarkers increased compared with single biomarker predictors, specificity was reduced.

A promising biomarker with limited predictive data reported in one study was proteins and cytokines found in the placenta.[56] Specifically, proteins that influence angiogenesis as well as pro-inflammatory and anti-inflammatory cytokines were evaluated in a group with a history of PAE. The study only provided information on AUC, which reflects the integration of sensitivity and specificity characteristics; however, these data were in the fair to excellent range (range, 0.70–1.00; median, 0.79). In contrast to previous biomarker data, integration of different predictors resulted in improved prediction. Combined analysis of the levels of three proteins (i.e., ANX-A4, CCM-3, and VEGFR2) yielded an AUC of 1.00, and a combined analysis of another six proteins (VEGFR1, angR, VEGF-A, VEGF-C, VEGF-D, and beta-fibroblast growth factor) resulted in an AUC of 0.94. Combined cytokine levels also had good to excellent AUC values, with six pro-inflammatory cytokines (IL-1-beta, IL-2, IL-8, IL-12p70, interferon-gamma, and tumor-necrosis factor alpha) yielding an AUC value of 0.92 and four anti-inflammatory cytokines (IL-4, IL-6, IL-10, and IL-13) resulting in an AUC value of 0.83.[56]

Finally, circulating microRNAs (miRNAs) in maternal blood, which reflect epigenetic changes in response to PAE, have been explored as a potential biomarker in a sample of Ukrainian mother-infant dyads.[57] Levels of miRNAs were compared among pregnant women without PAE; pregnant women with heavy PAE whose children were impacted; and pregnant women with heavy PAE whose children were not impacted in either growth, dysmorphology, or brain development. Heavy PAE was defined as weekly heavy episodic or binge drinking (i.e., five or more standard drinks), five or more episodes of three to four standard drinks, or 10 episodes of one to two standard drinks. Impact of PAE on the offspring was assessed by trained physicians who completed a dysmorphology assessment and by psychologists who completed a neurodevelopmental evaluation with the child. Several miRNAs (n = 21) were identified as differing between the exposed–affected group and both other groups, and a random forest analysis was used to predict group membership while controlling for other group differences (i.e., maternal smoking). Seven of the top 10 variables retained in the initial predictive model were miRNAs. The most common miRNAs identified were likely to influence downstream pathways related to fetal and placental growth. Specificity was excellent (91%) and sensitivity (82%) was good for miRNA levels obtained in pregnancy; however, both specificity (74%) and sensitivity (77%) were only fair for changes in the miRNA levels over the course of the pregnancy. Although this was only one study, the findings suggest that assessments of levels of specific miRNAs obtained in pregnancy may improve sensitivity in predicting PAE-related outcome compared with other biomarkers that could be obtained in pregnancy.

Screening Tools

Screening tools were divided into five types of assessments, including facial features, neurophysiological responses in infants and older children, neuroimaging, questionnaire responses, and performance measures (see Appendix 3). In some cases, combinations of facial data and performance measures were used in predictive modeling; these are included in the performance measure section of Appendix 3.

Facial features

Eight studies have explored facial features as key predictors of an FASD-related diagnosis using in-person measurements and two-dimensional (2D) and three-dimensional (3D) photographs.[58-65] Specificity values were variable, ranging from poor to excellent, with only a couple of studies reporting levels in the fair to poor range (range, 43%–100%; median, 86%). Sensitivity levels also were in the good to fair range (overall range, 43%–100%; median, 92%), with the exception of one study where sensitivity using the facial analysis software of 2D pictures was in the poor range. Accuracy for prediction was typically in the fair to good range (range, 79%–100%; median, 93%). Advancing technology from in-person measurement to 3D computerized configural methods did not necessarily result in improved predictive characteristics, but comparisons are complicated because samples were from different countries (i.e., United States, South Africa, Germany, and Finland), and different methods were used for defining the outcome (variations of FAS and FASD, heavy alcohol-exposed) and reporting predictive results.

More recently, one study evaluated the use of a schema that coded alterations to ocular development to differentiate individuals with a clinical diagnosis of FASD.[66] The coding schema captured elements of visual acuity, refraction, strabismus/binocular function, and ocular structural abnormalities, with each area being coded from 1 to 4. Cut-off values of the total score (10 and 9) were evaluated relative to healthy controls; children with attention-deficit/hyperactivity disorder (ADHD);

children who were born prematurely (moderate to late); and children with Silver-Russell syndrome, a genetic condition with growth impairment and neurodevelopmental compromise.[67] Similar to attempts to capture facial features, specificity was good to excellent (88%–100%), but sensitivity was poor (43%–57%). AUC estimates were variable, ranging from 0.60 to 0.92, with the higher estimate reflecting comparisons to healthy controls.

Infant neurophysiology

Early identification of alcohol-related brain impairment has been attempted using indices of infant neurophysiological responses, including eye-blink conditioning[68] and cardiac orienting response (COR).[69,70] These procedures use physiological responses in the context of a learning paradigm that can be implemented with infants. For eye-blink conditioning, classical conditioning is used where an unconditioned stimulus (i.e., puff of air) that elicits a reflexive eye blink is paired with a conditioned stimulus (i.e., auditory tone or picture) over repeated trials. After many pairings, the conditioned stimulus is then able to elicit the eye-blink response. Rate of learning is assessed by the percentage of pairing trials of the conditioned stimulus with the unconditioned stimulus needed before the eye blink is elicited by the conditioned stimulus in the absence of the unconditioned stimulus. In the case of COR, heart rate responses are monitored while stimuli (i.e., auditory tone or picture) are presented over several trials, referred to as habituation trials, and then after presenting novel but similar stimuli over several trials (dishabituation trials). Heart rate typically decelerates in response to novel information and returns to baseline over the course of several habituation trials; it decelerates again in response to the second novel stimulus. The magnitude of the deceleration in the first three habituation trials is believed to reflect the infant's encoding of stimuli, whereas the magnitude of the first three dishabituation trials reflects the infant's ability to differentiate the first and second related stimuli, indexing early memory functioning. These methods are advantageous as standardized early assessments of cognitive functioning often are not adequate in assessing alcohol-related brain impairment.

Eye-blink conditioning was reported in one study that provided data for its predictive utility relative to FAS and to a broader spectrum of individuals with heavy PAE, defined as averaging at least 1.0 oz absolute alcohol per day or ≥ five standard drinks per occasion in the first trimester of pregnancy; and a group defined as having FASD.[68] Eye-blink conditioning had a sensitivity of 100% for FAS prediction, but this fell to 70% for prediction of a broader spectrum of heavy PAE and FASD. Specificity was comparable for both predictive models at 75%. Overall accuracy was 82% for predicting FAS and 72% for predicting heavy PAE/FASD. The PPV value was 87% for heavy PAE/FASD and 63% for FAS alone, and NPV was 51% for predicting heavy PAE/FASD and 100% for FAS alone.

Findings for COR were not reported in terms of sensitivity, specificity, and overall accuracy but were reported in terms of PPV, NPV, and AUC values in two different articles using overlapping samples of Ukrainian mother-infant dyads.[69,70] Using the key features of COR (i.e., speed of the response, average trough), a PPV of 82%, an NPV of 62%, and an AUC value of 0.81 were reported in one of the studies for predicting neurodevelopmental impairment at 12 months.[70] Only small incremental gains were obtained when including maternal drinking information in the model. In the second study, an index score derived from the visual COR data had an AUC value of 0.77 for predicting later preschool FASD status.[69] These results suggest that early neurophysiological responses may be useful in improving identification of individuals with neurodevelopmental impairment in infancy, which has often been a key factor limiting early diagnosis.

Neurophysiology with older children

Neurophysiological responses assessed in older children have included auditory evoked potentials and eye-tracking or saccadic eye movements. One study evaluated auditory evoked potentials, which assess the time it takes for a signal to travel along the auditory nerve track in response to sound stimuli.[71] Auditory evoked potentials by themselves had fair sensitivity (79%) and poor specificity (43%) and overall accuracy (61%). However, when various indices of P300 responses were combined (e.g., latency, magnitude), increased differentiation of individuals with FASD from individuals with Down syndrome was found (sensitivity, 79%; specificity, 86%; and overall accuracy, 82%).

Eye-tracking movements also have been used to identify children impacted by PAE.[72] Two studies provided data regarding predictive validity of eye-tracking measures in individuals with FASD.[73,74] Accuracy ratings ranged from poor (65%) to excellent (90%). Combining eye-tracking information with data obtained from diffusion tensor imaging and neurobehavioral testing resulted in improved accuracy in one study (range of 65%–76% improved to 85%).[73] Eye-tracking movements also have been used to predict the impact of other neurodevelopmental disorders,[75,76] suggesting the importance of studies that attempt to establish differential predictive validity for the effects of PAE relative to other neurodevelopmental disorders (e.g., autism). This likely is also true of the infant neurophysiological measures (i.e., COR and eye-blink conditioning), which also have been used to determine mean group differences between other clinical groups and typically developing controls.[77,78]

Neuroimaging

Three neuroimaging studies provided predictive data for the impact of PAE.[73,79,80] Using weighted volumetric scores of specific brain regions, specificity was good (88%), but sensitivity was still in the poor range (64%).[80] The combination of four key features of diffusion tensor imaging also provided relatively

poor accuracy (67%) in predicting an FASD diagnosis.[73] Excellent specificity (95%) was reported for measurement of the "hook" area of the corpus callosum, but sensitivity of this measurement was poor (52%), suggesting that this method did not identify those impacted by PAE at better than chance levels.[79] This suggests that, like other biomarker prediction of PAE and PAE impact, prediction based on neuroimaging findings provides a clear signal of PAE or its impact, but is not sufficiently sensitive to capture the range of impact commonly seen in individuals exposed to alcohol.

Parent questionnaire measures

Six identified studies reported predictive characteristics of caregiver or provider responses to a questionnaire in identifying children with alcohol exposure or FASD.[81-86] Parental responses to questionnaires developed specifically for identifying children impacted by PAE or standardized measures used to flag aspects of alcohol teratogenesis typically had good to excellent specificity (overall range, 66%–96%; median, 83%); only one study using subsets of items from the Child Behavior Checklist yielded sensitivity in the poor to fair range.[82] Sensitivity reported in these studies was poor to excellent (range, 54%–100%, median, 85%), with the lowest sensitivity reported in a study attempting to differentiate only pFAS in one analysis (54%).[85] Relatively few studies reported overall accuracy rates, which ranged from poor to excellent (range, 68%–94%; median, 71%). The wide range in predictive characteristics of these types of data was dependent on the definition of the predictor (PAE, pFAS, FAS, or FASD) and the comparison group used—typical healthy controls or controls with ADHD. Incomplete evaluation of those who screened negative also may have overinflated estimates in one study of the predictive characteristics as this method fails to include the possibility of false negatives in the screening process.[84]

Child performance measures

Nine studies identified predictive characteristics of child performance measures and combinations of performance measures and other indicators of PAE or FASD.[87-95] These ranged from quick screening tests to complex neurobehavioral batteries in isolation or in combination with dysmorphology information. Of these nine studies, one assessed the predictive characteristics of motor assessments,[92] whereas another two studies looked at aspects of narrative speech only.[94-95] Specificity ratings for all nine studies ranged from poor (45%) to excellent (100%), and sensitivity ratings ranged from poor (2%) to excellent (100%). Overall accuracy in these studies also ranged from poor (49%) to excellent (100%). Two of the nine studies compared individuals with PAE to both typical healthy control groups and to other clinical groups separately or in combination with the healthy control group.[89,90]

Discussion and Conclusions

Identifying children who have been prenatally exposed to alcohol or, more importantly, have been negatively impacted by their exposure continues to be an important area of investigation. Although a range of biomarkers and screening tools have been explored, there is no agreed-upon procedure or method that provides excellent sensitivity, specificity, and overall accuracy, suggesting the need for continued research. A general theme found in the existing literature is higher specificity then sensitivity for many of the biomarkers and screening tools used to identify PAE and its impact. This means that although researchers and clinicians often have confidence when they identify PAE or its impact, they struggle with capturing the full range of individuals impacted. Exceptions to this were found in recent investigations of biomarkers of PAE using miRNAs related to growth and vascular development,[57] proteomic changes associated with PAE,[56] and combinations of markers estimating levels of various cytokines.[56] However, replications of these findings across other samples are needed to confirm the limited data currently available on the predictive characteristics of these biomarkers.

For predicting the outcomes of alcohol teratogenesis, facial features operationalized using varying methods (i.e., in person, 2D, or 3D) provided relatively high sensitivity, specificity, and accuracy, but a few point estimates were less effective. Neurophysiological responses assessed in infancy and later childhood were able to differentiate individuals impacted by PAE, but the upper limits of prediction were in the fair to good range. Moreover, there was some indication that these responses were better at defining pFAS/FAS rather than the full spectrum of FASD, including heavy PAE. Neuroimaging methods, including volumetric and diffusion tensor imaging, also had high specificity but poor sensitivity, similar to biomarkers of PAE alone. Parent and professional responses to questionnaires had both good sensitivity and specificity, with the exception of one comparison that attempted to discriminate specific subgroups of FASD. This increased sensitivity relative to other biomarkers and screening tools may be biased by the fact that all studies in this area involved clinical FASD samples, which may reflect shared variance associated with the parent seeking treatment for the child. Replications in prospective cohorts of exposure may be helpful in clarifying this potential bias in predictive validity. Child performance measures had varying ranges of success in predicting those impacted by PAE, which seemed to vary as a function of inclusion of other biomarkers and the nature of the comparison sample utilized in the prediction.

Limitations in the Existing Literature

The definition of the criterion to be predicted was problematic across studies. Maternal report of PAE or heavy PAE was operationalized using multiple different methods that were

integrated in different ways (e.g., summed, any positive response, principal component analysis of several responses). Moreover, results appeared to vary as function of the context in which the maternal self-report was collected. In one study, maternal self-report of PAE was higher than PAE confirmed using biomarker data.[52] In another study in the context of a health care environment, however, estimates of PAE using these methods were in the opposite direction.[96] Even in studies of FAEE levels that were conducted in the same hospital setting where participants were assured of confidentiality, FAEE levels were dramatically higher when they were sampled from de-identified meconium, which did not require maternal consent, than when informed consent from the mother was needed.[97] Mothers with the heaviest prenatal alcohol use were more likely to self-select out of the study,[98] most likely in response to the stigma associated with PAE.[99]

A number of studies used other biomarkers to validate a novel biomarker. Convergent validity is useful in verifying the validity of the novel biomarker but limits the window of detection between biomarkers; moreover, threshold or cutoff values used to signal a positive test also varied. Often biomarkers reflect severe alcohol use disorder as they are indicators of damage to organs (e.g., liver) over a prolonged period; however, these methods often failed to capture the full range of FASD or PAE that can have adverse impact on a developing fetus. Other biomarkers are byproducts of the metabolism of alcohol and have limited windows for detecting PAE. For each biomarker, other factors also may reduce the validity of their prediction, including personal care and hygiene (e.g., corruption from chemicals used in hair and nail care), other foods that may produce alcohol metabolites during decomposition,[97] and willingness of the mother to provide the biological sample. Some investigators have opted to use a combined approach, although costly, to predict PAE status[35,45,53] to compensate for the individual weaknesses or limitations of any one method of identification of PAE.

Many studies used an FAS or FASD diagnosis as the outcome, but diagnostic formulations used in the field vary considerably, and evidence suggests that the degree of agreement across methods is low.[12] The development of a consensus diagnostic formulation for individuals with FASD would be helpful in reducing error variance associated with the diagnostic formulations. As mentioned previously related to parental questionnaires as screening tools, use of clinical samples also is biased because it selects for individuals who sought care for the treatment of the child. This can result in circularity in defining the screening tool as the predictor when the screening tool may be drawn from the same construct domain or type of test used to categorize or diagnose the clinical group. Implementation of screening approaches across multiple samples—including both clinical and prospective cohorts of PAE from diverse populations that vary in ethnic, geographic, and cultural backgrounds—may help with eliminating these biases.

Another limitation of some studies was that they provided predictive estimates but failed to sample the criterion within the entire pool of individuals screened.[59,84] This approach occurred in larger screening cohorts where individuals who screened negative were not sampled further and were assumed to be true negatives. These assumptions may result in overestimation of the predictive characteristics of the biomarker or screening tool.

Sensitivity and specificity characteristics are independent of the prevalence of the condition under investigation (e.g., PAE), but accuracy, PPV, and NPV are influenced by the rate of PAE or individuals impacted by PAE in a study's sample (see Figure 1 for computational formulas). Considerable variation existed across studies in the ratios of affected and nonaffected individuals in the sample. In many studies, both groups were comparable in size, which results in an estimate of the predictive characteristics under circumstances where the prevalence of the condition in the sample is substantially higher than the rate anticipated in the general population. Changes in the sensitivity and specificity of a biomarker if the prevalence of the condition deviates from 50% can result in reduced validity of estimates of the overall accuracy of a biomarker or screening tool.[100] This suggests that the accuracy ratings commonly found for biomarkers of PAE and its impact may be overweighted by their high specificity and that these biomarkers are less predictive in real-world settings where the prevalence has been estimated to fall between 5 to 50 per 1,000 children.[18,19] Implementation of biomarkers or screening tools in clinical trials in the context in which they are intended to be used may help to evaluate the true accuracy of these tools.

The studies surveyed also differed in comparison samples used, with some studies including typical healthy controls and others attempting to differentiate offspring with PAE relative to other clinical groups who might present for diagnosis. Estimates of predictive validity of biomarkers or screening tools relative to typical healthy controls are often higher than those found when using a clinical comparison group. However, the latter approach provides a better estimate of the usefulness of a biomarker or screening tool to clinicians asked to determine if a given child has been impacted by their PAE. In evaluating biomarkers or screening tools, researchers should consider a tiered approach with a first evaluation relative to typical controls, followed by evaluation relative to other clinical groups to improve understanding of the clinical utility of the biomarker or screening tool. The final tier would then involve an actual clinical trial of the clinical utility of the biomarker or screening tool and an assessment of where it fits within a clinical diagnostic algorithm—that is, whether it functions more as a screener that can flag the need for other diagnostic assessments or as an actual diagnostic tool, indicating its high concordance with the clinical endpoint.

Finally, the scalability of a biomarker or screening tool is also important to consider. The financial cost of the assay or test and the expertise needed to carry out an assessment can

dramatically limit the utility of a given biomarker or screening tool, particularly in countries with low resources. The gold standard for diagnosis is a multidisciplinary team assessment that includes at a minimum a physician who can assess alcohol-related dysmorphology and a psychologist who can assess neurobehavioral impairment. Even if variations in diagnostic criteria utilized among existing clinics are resolved, this method of identification in no way can meet the needs of those impacted by PAE given the recent prevalence estimates. This is true in countries with considerable resources as well as in those with minimal resources. Therefore, when designing biomarkers or screening measures, it is important to consider to what extent the test can be implemented globally with limited expense and expertise.

Limitations of This Review

This review was not intended to be a comprehensive review of each biomarker as several studies were eliminated that characterized biomarkers in different populations, established group differences, or estimated costs associated with implementation. Several existing reviews have provided in-depth discussions of one or more biomarkers or screening tools with greater details on the ease of collection, detection windows, limits of detection, costs, and feasibility of use.[101-104] This article aimed to focus on the predictive characteristics of biomarkers and screening tools to assess PAE and its associated impact. The search process using the selected terms may have missed relevant articles as several additional papers were found among the references in those articles identified using the initial search terms. Also, most biomarkers did not have sufficient numbers of studies for a true meta-analysis given the variation in threshold or cutoff values used to define risk and in the predictor. As a result, the range and median value of data obtained from the articles were provided. Providing uniform data-reporting formats in future studies would help with subsequent attempts to integrate these types of studies.

Future Directions

The relative importance of the predictive validity characteristics depends on the goals of the screening and on the diagnostic algorithm in which the biomarker or screening tool is being used. PPV and NPV only incorporate validity of a positive or negative test signal, respectively, and are most useful for clinicians trying to interpret a biomarker or screening tool result relative to a clinical endpoint. Accuracy provides a summary of the overall correctness of the biomarker or screening tool, but does not fully capture its errors (i.e., false positives and missed cases). In cases where the costs of these errors are high, accuracy is an inadequate indicator of success. One could argue that this is the case for PAE and its associated impact, where false positives could potentially be stigmatizing and missed cases would limit opportunities for harm reduction and intervention during early periods of neuroplasticity. Many biomarkers and screening tools related to PAE have good specificity, but their implementation requires further evaluation of the cost-benefit ratios of use within given environments and discussions regarding the ethics of implementation relative to patient privacy and autonomy. Much progress is needed in the development of biomarkers and screening tools to improve sensitivity, which is likely to be most valued by individuals affected by PAE and those who care for them as low sensitivity results in lost opportunities for harm reduction and early intervention. AUC values provide a tool for estimating predictions that capture both sensitivity and specificity elements but may obscure relative weaknesses in one or the other. Ultimately, final decisions on clinical implementation should include input from key stakeholders who may assign different value judgments to these predictive characteristics.

Improvements in the predictive characteristics of biomarkers and screening tools would have important ramifications for surveillance methods and clinical care of individuals negatively impacted by PAE. Surveillance methods that use biomarkers or screening tools currently are limited by the low sensitivity of most available biomarkers and screening tools because a negative test result does not exclude individuals who may be negatively impacted by PAE. Surveillance studies that assume those who screen negative are unaffected and do not conduct further evaluations therefore may be underestimating true case prevalence rates. The clinical use of biomarker or screening tools also has been limited by insufficient data on predictive utility characteristics in published studies. Moreover, implementation within clinical environments often only takes place if researchers are exploring the use of the biomarker or screening tool in their studies. Improved reporting of the predictive validity characteristics of these measures are needed before consensus could be reached to support larger-scale implementation of these biomarkers and screening tools.

The field of alcohol teratogenesis initially sought to determine if PAE resulted in group differences from offspring not exposed to PAE on a variety of outcomes; however, future efforts also need to include efforts to help identify affected individuals. Predictive validity information moves beyond mean group differences and attempts to determine if a given measure's dispersion is such that a threshold, cutoff value, or rule based on an outcome or a cluster of outcomes could be used to identify those impacted by PAE. In most cases, these differing aims could be achieved within the same study, using different analyses to help with identifying better biomarkers that can improve early identification and access to habilitative care.

There are many promising areas where group differences have been explored but predictive characteristics have not yet been reported. One promising diagnostic tool may involve functional near-infrared spectroscopy,[105,106] which assesses changes in oxygenation levels of brain tissue by shining near-infrared light through the scalp that is then

differentially reflected back to a sensor as different light wavelengths depending on whether or not the blood is oxygenated. Individuals with FASD show specific patterns of buildup of deoxygenated hemoglobin over time in response to prefrontal cortex activation that differ both from typically developing children and from those with other neurobehavioral impairments. Epigenetic changes, including DNA methylation, histone modifications, and other miRNAs associated with PAE, may also be effective biomarkers,[107,108] although diagnostic analyses of these measures have rarely been reported. One promising study assessing changes in DNA methylation (i.e., the process of adding methyl groups to a DNA molecule) found that children with PAE and their mothers both had higher DNA methylation levels of proopiomelanocortin and PER2, a gene involved in regulating circadian rhythms, resulting in reduced expression of these genes. In contrast, postnatal choline supplementation, which increases the bioavailability of additional methyl groups after birth, resulted in reduced DNA methylation and increased expression levels of these stress-regulatory genes.[109] In addition, the health consequences of PAE are just beginning to be explored, and it may be important to determine to what extent these consequences may help identify individuals impacted by PAE.

Going beyond group differences to establish the diagnostic test validity of an outcome relative to healthy children without PAE and then relative to other children with other neurobehavioral conditions will provide the needed information to evaluate effectively whether these potential biomarkers will have clinical utility and should be further evaluated in the context of a biomarker clinical trial. This transition to a systematic process of biomarker and screening tool evaluation is needed to address the public health need of improving early identification of individuals impacted by PAE so that harm reduction and habilitative care efforts can be implemented.

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Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
Azurmendi-Funes et al., 2019[46]	Mothers at a public maternity hospital in Spain	20 High risk due to PAE (20 g alcohol or ≥ 3 binges of ≥ 40 g alcohol)	71 Non–high-risk PAE	%CDT (cutoff ≥ 95%)	Maternal blood	Self-report of PAE	25.0	93.0		81.5		.75/.71
				AST								.47
				ALT								.54
				GGT								.42
				MCV								.38
Bakdash et al., 2010[38]	Meconium samples	116 FAEE (≥ 100 ng/g)	480 FAEE (< 100 ng/g)	EtG ≥ 500 ng/g	Meconium	FAEE > 100 ng/g	78.6	98.7	82.5	98.4	97.3	
Bakhireva et al., 2014[35]	Women from a specialty treatment clinic	28 PAE	32 No PAE	PEth > 8 ng/mL delivery	Maternal blood	Self-report of PAE	22.2	100				
				GGT > 40 U/L mid-gestation			14.8	100				
				GGT > 40 U/L delivery			10.7	86.2				
				%CDT > 2.0% mid-gestation			3.7	100				
				%CDT > 2.0% delivery			10.7	96.7				
				EtG ≥ 25 ng/mL mid-gestation	Maternal urine		14.8	96.9				
				EtG ≥ 25 ng/mL delivery			7.4	96.9				
				EtS ≥ 7 mid-gestation			14.8	100				
				EtS ≥ 7 delivery			7.4	96.9				
				PEth (> 8 ng/mL)	Infant blood spots		32.1	100				
				FAEE (sum of 9)	Infant meconium		28.6	81.3				
				All mid-gestation			32.1	96.9				

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
				All delivery + infant PEth (mid-gestation/ both)			50/50	81.3/93.8				
Bearer et al., 1999[34]	Mothers and infants from a large urban hospital enrolled in a study on the effects of cocaine	127 Positive FAEE	92 Negative FAEE	FAEE sample peak at 0.02 minutes/ standard for ethyl linoleate	Meconium	Periconceptual self-report of PAE (variation in dosage level of self-report)	64–81	43–48				
						Trimesters 2 and 3 self-report of PAE (variation in dosage level of self-report)	66–72	66–72				
Bearer et al., 2003[36]	Mothers and infants from South Africa	27 Meconium samples with varying levels of ethyl linoleate	None	FAEE ethyl oleate ≥ 32 ng/g	Meconium	Self-report of PAE (≥ 1.5 AA/drinking days)	84	83	94	63		.92
				FAEE ethyl oleate ≥ 13 ng/mg			100	66.7	91	100		
				FAEE ethyl oleate ≥ 77 ng/mg			68.4	100	100	50		
Bearer et al., 2005[28]	Postpartum women in large urban teaching hospital in Cleveland, Ohio (*n* = 248), and Muslim women receiving care at a hospital in Jordan (*n* = 30)	169 Nonabstainers	55 Abstainers/ nondrinkers (30 Jordan and 25 Cleveland)	FAEE score derived from principal component analysis of variance	Meconium	Self-report > 7 drinks per drinking day	78–88	59–63	7–18	98–99		.69–.75
						Maternal self-report > 21 drinks/drinking day	72–84	50–60	7–15	95–98		.60–.71

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
Cabarcos et al., 2014[39]	Infant meconium samples	FAEE ≥ 600 ng/mg; ethyl myristate, ethyl palmitate, ethyl stearate, and EtG	FAEE < 600 ng/mg; ethyl myristate, ethyl palmitate, ethyl stearate, and EtG	FAEE ≥ 600 ng/mg; ethyl myristate, ethyl palmitate, ethyl stearate, and EtG	Meconium	EtG (> 50 ng/g)					61.7	
Chan et al., 2004[99]	Mothers from Toronto, Canada (*n* = 104) and Israel (*n* = 103)	6 Heavy PAE and 73 with FAEE above lower limit of detection	None	Women with AUD or FAEE ≥ 2 nmol/g	Meconium	Self-report and clinical referral of AUD	100	98	63	100		
Eichler et al., 2016[47]	Meconium samples from infants whose mothers were seen in pregnancy and when child was 6–8 y	43 Self-reported PAE in trimester 3	137 Self-reported no PAE	EtG > 10 ng/g	Meconium	Self-report of PAE in trimester 3	33.3	79.0	32.6	79.6	68.3	
				EtG > 120 ng/g	Meconium	Self-report of PAE trimester 3	18.6	86.9	30.8	77.3	70.6	
Ferraguti et al., 2017[48]	Women who attended a gynecology and obstetrics hospital in Rome	46 Alcohol drinkers	24 Abstainers	EtG > 500 ng/mL	Maternal urine	Self-report of PAE	40.9	75.0	60.0	58.1	58.7	
Gauthier et al., 2015[29]	Mothers of infants admitted to the newborn intensive care unit in a large inner-city hospital	11 "Drinkers" as defined by a positive AUDIT item	56 Nondrinkers	FAEE ethyl stearate	Placental FAEE	Maternal AUDIT+	82.0	87.0	50.0	97.0		.93

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
				FAEE ethyl linoleate			82.0	83.0	44.0	97.0		.88
				FAEE OLL			82.0	94.0	70.0	97.0		.91
				FAEE OLL + stearate			82.0	93.0	64.0	97.0		.92
Goecke et al., 2014[30]	Women attending outpatient visit at obstetrics and gynecology department at the University of Erlangen-Nuremberg, Germany, FRAMES	204 Self-reported PAE	782 Self-reported no PAE	Ethyl stearate > 0 ng/g	Meconium	Self-report of PAE	32.5	76.7	27.3	80.8	67.3	
				EtG ≥ 0 ng/g			26.96	85.7	33.7	81.4	73.3	
Gomez-Roig et al., 2018[49]	Pregnant women seen at a maternal fetal and neonatal medicine hospital	99 Gestational alcohol users	54 Abstinent women	EtG ≥ 7.0 pg/mg	Maternal hair	Self-report of PAE	2.0	96.3	50.0	37.0	37.3	
Gutierrez et al., 2015[41]	Biomarkers in Pregnancy Study Women attending UNM prenatal clinic providing care to women with SUD and addiction	42 PAE	43 No PAE	EtG ≥ 8 pg/mg	Maternal hair	Self-report of PAE (composite index)	19.1	86.1	57.1	52.1		
				GGT > 40 U/L			20	97.6	88.9	56.1		
				%dCDT > 2.0%			5	100	100	52.5		
				%dCDT > 1.7%			22.5	71.4	42.8	49.2		

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
				uEtG ≥ 25 ng/mL			4.9	97.6	66.7	51.2		
				EtS ≥ 7 ng/mL			7.3	97.6	75.0	51.9		
				PEth ≥ 8 ng/mL			17.5	100	100	56.6		
Himes et al., 2015[31]	Meconium samples from Safe Passages Study of the Prenatal Alcohol in SIDS and Stillbirth Network	58 Women who drank past 19 weeks in pregnancy	33 No PAE	Maternal self-report of PAE	Meconium	FAEE sums (4–9)	52–64.9	45.1–51.4				
						EtG (10–50 ng/g)	68.6–82.4	71.4–75.0				
						EtG (333 or 444 ng/g)	96.7–96.8	71.4–75.0				
						EtG or EtS (> 2.5 ng/g)	66.7–83.3	64.6–76.3				
						FAEE or EtG/EtS	70.4–75.0	61.7–78.4				
Holbrook et al., 2019[56]	UNM Clinic ENRICH Cohort (prenatal clinic for individuals with SUD)	13 PAE as defined by AUDIT Score ≥ 8 total consumption of ≥ 84 drinks, positive ethanol biomarker	13 No PAE	Angiogenesis-related proteins Western blot		PAE group via multiple determination						.78–.89
				Angiogenesis-related proteins Meso scale								.76–.81
				Pro-inflammatory cytokines								.70–.78
				Anti-inflammatory cytokines Meso scale								.70–.76
				ANX-A4, CCM-3, VEGFR2,								.100

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
				VEGFR1, angR, VEGF-A, VEGF-C, VEGF-D, beta-FGF								.94
				6 Pro-inflammatory cytokines								.92
				4 Anti-inflammatory cytokines Meso Scale								.83
Howlett et al., 2018[42]	Pregnant women from England seeking prenatal care in first trimester; Biosample study with no consent	20 Positive for GGT	567 Negative for GGT	CDT ≥ 1.60% and ≥ 1.87 for probable chronic alcohol use	Maternal blood	GGT ≥ 45 U/L	5.00	98.8	12.5	96.7	95.6	
Joya et al., 2016[50]	Mother-infant dyads from Spain	19 Self-reported some alcohol consumption in pregnancy	61 Self-reported no alcohol consumption	EtG (> 11 pg/mg)	Maternal hair	EtG meconium (≥ 30 ng/g vs. < 30 ng/g)	76.2–85.7	73.7–78.9				
Kwak et al., 2014[26]	Mothers from Korea who reported PAE	8 Heavy drinkers; 19 moderate, 85 light; FAEE for 54 for light/moderate	182 No PAE	FAEE ≥ 20 nmol/g	Meconium	Self-report of PAE	3.7	98.9	50.0	77.6	77.1	.52
				FAEE ≥ 2 nmol/g			22	73.6	20	76.1	61.7	
				FAEE ≥ 10 nmol/g			5.6	94.5	23.1	77.1	74.2	
Kwak et al., 2014[55]	Mothers from Korea who reported PAE	8 Heavy drinkers; 22 moderate, 87 light; contrasted moderate to heavy (n = 30)	188 No PAE Light drinkers = 87	PEth 4.2 nmol/L	Maternal blood	Maternal self-report of PAE	100	96.4				.99
				PEth 3.8 nmol/L			66.7	96.4				.85
				PEth 1.2 nmol/L			40.7	95.4				.69

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
Lamy et al., 2017[51]	Meconium samples collected from Rouen (Normandy)	20 Self-reported PAE	607 Self-reported no PAE	EtG > 60 ng/g	Meconium	Self-report PAE in trimester 3	5	97.4	5.9	96.9	94.4	
Maxwell et al., 2019[53]	Neonates born at a tertiary care center in Charleston, West Virginia	43 PEth positive	119 PEth negative	PEth DBS ≥ 8 ng/ml	Umbilical Cord	Self-report of PAE	4.7	98.3	50.0	74.1	73.5	
						Urine PEth	0.0	97.4	0.0	72.6	91.2	
						Mom peripheral blood PEth	3.9	9.0	12.5	74.0	69.2	
May et al., 2018[52]	Mothers from Western Cape province in South Africa	126 Self-reported PAE	67 Self-reported no PAE	PEth > 8 ng/g	Maternal DBS PEth/ Fingernail clipping (EtG)	Self-report prior 7 days	92.0	58.7	50.0		68.9	
				PEth > 8 ng/g		Self-report prior 21 days	72.1	83.0	91.2		75.1	
				EtG > 8 ng/g		Self-report prior 7 days	65.0	72.9	52.0		70.5	
				EtG > 8 ng/g		Self-report prior 21 days	50.7	92.5	94.7		62.2	
				One or both		Self-report prior 7 days	95.0	48.9	45.6		69.2	
				One or both		Self-report prior 21 days	80.0	75.5	89.6		78.8	
Ostrea et al., 2006[33]	Mother/infant dyads	93 Self-reported PAE	31 Self-reported no PAE	Ethyl linoleate > .05 µg/g	Meconium	Self-report any alcohol	26.9	96.8	96.2			.60
				Ethyl arachidonate > .20 µg/g			18.3	96.8	94.4			.57

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
Sarkola et al., 2000[45]	Women with a history of SUD who were attending a special outpatient clinic	44 used alcohol and drugs; 8 of 13 who drank heavily had child with FAE impact; 31 drank moderately	62 Controls	GGT	Maternal blood	Group status (heavy vs. moderate PAE)	30.8	75.6	40.0	71.0	63.4	.65
				MCV			15.4	100	100	73.8	75.0	.72
				CDT			7.7	93.6	33.3	70.7	68.2	.48
				CDT/transferrin			15.4	87.1	33.3	71.1	65.9	.54
				MCV and GGT			38.5	78.6	45.5	73.3	65.9	
				GGT	Maternal blood	FAE vs. other exposed (exposure and growth HC)	50.0	81.8	40.0	87.1	75.6	.76
				MCV			20.0	100	100	81.8	82.6	.87
				CDT			12.5	94.4	33.3	82.9	79.6	.60
				CDT/transferrin			25.0	88.9	33.3	84.2	77.2	.62
				MCV and GGT			62.5	81.8	45.5	90.0	78.1	
Stevens et al., 2020[54]	Pregnant women approached at antenatal clinic visits in Auckland, New Zealand	39 PEth exposure and 30 moderate to heavy exposure	26 No PEth	PEth > 8 ng/g	Infant DBS	Self-report No/Low vs. Heavy	63.0	42.1	43.6	61.5	50.8	
				PEth > 20 ng/g		Self-report No/Low vs. Heavy	52.4	45.7	36.7	61.5	48.2	
				PEth > 8 ng/g		TWEAK+	43.2	60.0	61.5	41.7	50.0	
				PEth > 20 ng/g		TWEAK+	54.6	46.9	41.6	60.0	50.0	

Appendix 1. Predictive Characteristics* of Biomarkers for Prenatal Alcohol Exposure Status (Continued)

Author	Subject Description	PAE/FASD Sample Description	Control Sample Description	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC From ROC
Wurst et al., 2008[40]	Mothers seeking routine ultrasound at university hospital	3 FAEE (≥ 2.3 pg/mg)	100 FAEE (< 2.3 pg/mg)	Hair EtG social drinking (≥ 7 pg/mg to ≥ 25 pg/mg)	Maternal hair	FAEE ≥ 2.3 pg/mg	0	87.5	0	96.6	84.9	
				Hair EtG heavy drinking (≥ 25 pg/mg)			0	95.5	0	96.6	92.3	

*Prediction characteristics evaluated in each study included sensitivity, specificity, NPV, PPV, accuracy, and AUC derived from ROC curves. Sensitivity refers to the probability that the test is positive when the condition is present. Specificity refers to the probability that the test is negative when the condition is not present. PPV refers to the probability that the condition is present when the test is positive. NPV refers to the probability that the condition is not present when the test is negative. Accuracy refers to the overall probability that the case is correctly classified from the test. Finally, AUC is derived from creating receiver operating curves by plotting the true positive rate (sensitivity) relative to the false positive rate (1-specificity). The area under the curve references the area on the graph created by the regression line relative to the chance rate of prediction. Values of 1 would indicate perfect condition, and values of 0.50 would indicate chance prediction using a yes/no model. Predictive validity values are presented as percentages with the exception of AUC values, which are reported in proportions of accurate diagnostic classification with values of 0 to 1.00.

Note: AA, absolute alcohol; ALT, alanine aminotransferase; angR, angR protein; ANX-A4, annexin-A4; AST, aspartate aminotransferase; AUC, area under the curve; AUD, alcohol use disorder; AUDIT, Alcohol Use Disorders Identification Test; beta-FGF, beta-fibroblast growth factor; CCM-3, cerebral cavernous malformation 3; CDT, carbohydrate-deficient transferrin; CDT/transferrin, carbohydrate-deficient transferrin to total transferrin ratio; DBS, dried blood spots; dCDT, disialo-carbohydrate-deficient transferrin; ENRICH, Ethanol, Neurodevelopment, Infant and Child Health; EtG, ethyl glucuronide; EtS, ethyl sulfate; FAE, fetal alcohol effect; FAEE, fatty acid ethyl ester; FASD, fetal alcohol syndrome disorders; FRAMES, Franconian Maternal Health Evaluation Studies; g, gram; GGT, gamma-glutamyltransferase; HC, head circumference; MCV, mean corpuscular volume; ng/g, nanograms per gram; ng/mg, nanograms per milligrams; ng/mL, nanograms per milliliter; nmol/g, nanomoles per gram; nmol/L, nanomoles per liter; NPV, negative predictive value; OLL, oleate + linoleate + linolenate; PAE, prenatal alcohol exposure; PEth, phosphatidylethanol; pg/mg, picograms per milligram; PPV, positive predictive value; ROC, receiver operating characteristic; SIDS, sudden infant death syndrome; SUD, substance use disorder; TWEAK+, TNF [tumor necrosis factor]-like weak inducer of apoptosis; uEtG, urinary ethyl glucuronide; µg/g, micrograms per gram. U/L, units per liter; UNM, University of New Mexico; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Appendix 2. Predictive Characteristics* of Biomarkers for Fetal Alcohol Spectrum Disorders and Associated Symptoms

Author	Sample Description	PAE/FASD	Control Sample	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
Andreu et al., 2019[27]	55 native Spanish children (ages 8–12) from the Meconium Project and 98 children adopted from Eastern European communities	55 native Spanish children with FASD; 33 FAEE > 2 nmol/g; of 98 children from Eastern Europe: 31 with FASD, 42 partial FAS, 6 ARBD, and 5 ARND	31 FAEE < 2 nmol/g	Serum IGF-II (below 5th percentile)	Infant blood	FASD diagnosis	12.7	100	100	14.8	24.2	
				Serum IGF-II (below 5th percentile)	Infant blood	FASD diagnosis	39.0	96.8	98.8	19.4	46.6	
Balaraman et al., 2016[57]	Ukrainian pregnant mothers at prenatal care visits	22 Heavy alcohol-affected	23 No PAE	Selected mRNAs mid to late pregnancy	Maternal blood	Defined by self-report and child outcome	81.8	91.3	90.0	84.0	86.7	
				Change in mRNAs			77.3	73.9	73.9	77.3	75.6	
Lee et al., 2018[32]	Meconium samples from infants born in hospitals—Uijeongbu St. Mary's Hospital, Catholic University of Korea	5 with HC and length ≤ 10th percentile	FAEE < 0.5 nmol/g	FAEE 0.5 nmol/g	Meconium	Growth delay—HC, length < 10th percentile	40.0	92.8			68.0	
			FAEE < 2 nmol/g	FAEE 2 nmol/g	Meconium		20.0	98.0			68.0	
			FAEE < 0.5 nmol/g	FAEE 0.5 nmol/g	Meconium		50.0	92.8			79.0	
			FAEE < 2 nmol/g	FAEE 2 nmol/g	Meconium		25.0	98.0			79.0	

Appendix 2. Predictive Characteristics* of Biomarkers for Fetal Alcohol Spectrum Disorders and Associated Symptoms (Continued)

Author	Sample Description	PAE/FASD	Control Sample	Biomarker	Source of Biomarker	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
Niemelä et al., 2016[43]	All live births in Finland from 1987 to 2005 were matched to a prenatal serum specimen biobank and compared to FAS in Finnish Register of Congenital Malformations	385 Mothers of children with FAS + 95 PAE but no FAS	745 Controls (mothers no PAE and no child effects)	CDT ≥ 1.79%	Maternal blood	Self-report/ FAS diagnosis	39.5/4.2	96.4	3.6			.78/.53
				GGT ≥ 40 U/L			41.0/13.7	95	5			.82/.72
				Combo CDT–GGT ≥ 3.35			54.0/14.7	95.3	4.7			.87/.73
				EtG ≥ 0.1 mg/L			16.9/6.1	100	0			.58/.56
Niemelä et al., 2016[44]	All live births in Finland from 1987 to 2005 were matched to a prenatal serum specimen biobank and compared to FAS in Finnish Register of Congenital Malformations	385 Mothers of children with FAS	745 Controls (mothers no PAE and no child effects)	CDT ≥ 1.79%	Maternal blood	FAS diagnosis	12.5	99.3	90.6	68.7	69.7	
				GGT ≥ 40 U/L			33.0	96.4	82.5	73.6	74.8	
				Combo CDT–GGT ≥ 3.35			33.5	98.0	89.6	74.0	76.0	
				EtG ≥ 0.1 mg/L			16.9	100	100	70.0	71.7	

*Prediction characteristics evaluated in each study included sensitivity, specificity, NPV, PPV, accuracy, and AUC derived from ROC curves. Sensitivity refers to the probability that the test is positive when the condition is present. Specificity refers to the probability that the test is negative when the condition is not present. PPV refers to the probability that the condition is present when the test is positive. NPV refers to the probability that the condition is not present when the test is negative. Accuracy refers to the overall probability that the case is correctly classified from the test. Finally, AUC is derived from creating receiver operating curves by plotting the true positive rate (sensitivity) relative to the false positive rate (1-specificity). The area under the curve references the area on the graph created by the regression line relative to the chance rate of prediction. Values of 1 would indicate perfect condition and values of 0.50 would indicate chance prediction using a yes/no model. Predictive validity values are presented as percentages with the exception of AUC values, which are reported in proportions of accurate diagnostic classification with values of 0 to 1.00.

Note: ADHD, attention-deficit/hyperactivity disorder; ARBD, alcohol-related birth defects; ARND, alcohol-related neurodevelopmental disorder; AUC, under the curve; CDT, carbohydrate-deficient transferrin; EtG, ethyl glucuronide; FAEE, fatty acid ethyl esters; FAS, fetal alcohol syndrome; FASD, fetal alcohol syndrome disorders; GGT, gamma-glutamyltransferase; HC, head circumference; IGF-II, insulin-like growth factor-II; mg/l, milligrams per liter; mRNAs, messenger RNAs; nmol/g, nanomoles per gram; NPV, negative predictive value; PAE, prenatal alcohol exposure; PPV, positive predictive value; ROC, receiver operating characteristic; U/L, units per liter.

Appendix 3. Predictive Characteristics* of Other Screening Tools for Fetal Alcohol Spectrum Disorders and Its Associated Symptoms

Author	PAE/FASD Sample	Comparison Sample	Predictor	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
Aring et al., 2021[66]	37 Children with FASD	65 Healthy children, 33 ADHD, 57 Moderate to late prematurity	FASD Eye Code ≥ 10	FASD	43	94				0.78
		65 Healthy children only			43	100				0.87
		16 Silver-Russell syndrome			43	88				0.6
		65 Healthy children								0.92
		All comparison groups								0.76
		33 ADHD								0.66
		57 Moderate to late prematurity								0.75
		65 Healthy children only	FASD Eye Code ≥ 9		57	98				0.87
Astley & Clarren, 1996[58]	42 FAS: 21 development sample and 21 validation sample	84 without FAS (including 4 with other genetic conditions) placed into 2 groups; 42 per group for development and then validation	Facial features: 2D continuous measurements philtrum/lip	Gestalt FAS	100	93				
			Likert scale rating philtrum & lip		100	100				
			Likert scale philtrum/lip continuous		100	100				
Astley et al., 2002[59]	Sampled 600 children in foster care screened	Facial analysis software	Facial features: 2D clinical FASD diagnosis	Screened + Gestalt FAS	100	99.8	85.7	100	99.8	
Moore et al., 2001[60]	41 FAS & 59 pFAS	31 Controls	Facial features: 6 2D craniofacial measurements	Clinical FAS/pFAS	98	90			96	
			2 Craniofacial measurements		100	100			100	
			5 Craniofacial measurements		86	94			88	
Widder et al., 2021[61]	22 FASD	31 Controls; 15 ADHD; 20 AUD/OUD; 18 depression	Facial features: 2D German BSI-FASD	Clinical FASD	77	70–100				
			Facial analysis software		67	44–79				
			German BSI-FASD adapted scoring		86	70–100				

Appendix 3. Predictive Characteristics* of Other Screening Tools for Fetal Alcohol Spectrum Disorders and Its Associated Symptoms (Continued)

Author	PAE/FASD Sample	Comparison Sample	Predictor	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
Fang et al., 2008[62]	50 Finnish FASD diagnosis	32 Finnish controls	3D facial coordinates	FASD	88.2	100	100	83.3	92.6	
	36 FAS Cape-Colored	31 Finnish controls			91.7	90	91.7	90	90.9	
	86 Combined FASD	63 Combined controls			82.8	76.2	82.8	76.2	80	
Mutsvangwa et al., 2010[64]	4 FAS (age 5)	11 Controls (age 5)	3D facial coordinates	Clinical FASD	80	100	100	90.9	93.3	
	13 FAS (age 12)	6 Controls (age 12)			90.9	62.5	76.9	83.3	79	
Suttie et al., 2017[63]	22 FAS and 75 heavy AE South Africans (ages 6–18) & 35 FAS and 73 heavy AE Caucasians from CIFASD (ages 3–18)	69 South Africans (ages 6–18) who were Cape-Colored & 141 Caucasians from CIFASD (ages 3–18)	3D facial curvature coordinates of face	FAS or heavily AE						0.95–0.98
			3D facial curvature coordinates of profile							0.82–0.96
			3D facial curvature coordinates of eyes							0.92–0.95
			3D facial curvature coordinates malar							0.90–0.95
			3D facial curvature coordinates of mandible							0.85–0.93
			3D facial curvature coordinates of nose							0.86–0.95
			3D facial curvature coordinates of lip vermillion							0.69–0.84
			3D facial curvature coordinates of philtrum							0.70–0.90
Valentine et al., 2017[65]	36 FAS	50 Controls	Facial dysmorphology novel analysis technology computer scoring	FAS	78	92	88	85		0.95
	31 pFAS			pFAS	79	78	67	87		0.82
	22 ARND			ARND	50	92	70	83		0.84

Appendix 3. Predictive Characteristics* of Other Screening Tools for Fetal Alcohol Spectrum Disorders and Its Associated Symptoms (Continued)

Author	PAE/FASD Sample	Comparison Sample	Predictor	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
	89 FASD			Any FASD	89	69	83	78		0.86
	36 FAS	50 Controls	Facial dysmorphology novel analysis technology manual scoring	FAS	99	89	87	99		0.96
	31 pFAS			pFAS	76	89	81	86		0.89
	22 ARND			ARND	43	92	70	79		0.74
	89 FASD			Any FASD	87	77	87	77		0.88
Jacobson et al., 2008[68]	12 FAS, 18 pFAS, 29 heavy PAE	20 Nonexposed controls; 4 nonexposed microcephalic	Physiological neural response: % criteria for eye-blink conditioning	FASD	70.2	75	87	51.4	71.6	
	10 FAS				100	75	62.5	100	82.4	
Kable et al., 2021[69]	26 PAE no diagnosis, 19 ARND, 5 FAS/pFAS	70 No PAE/no diagnosis	Physiological neural response: cardiac orienting response auditory COR Deviation Index	FASD						0.65
			Visual COR Deviation Index							0.77
Mesa et al., 2017[70]	Sample of Ukrainian infants with 26 having mild developmental delay	Sample of Ukrainian infants with 98 within normal limit development	Physiological neural response: cardiac orienting response Standard COR	12-month Bayley < 85			66	85		0.81
			Key features COR				62	82		0.81
			Maternal drinking				49	75		0.68
			Maternal drinking + standard COR				65	87		0.84
			Maternal drinking + key COR				62	80		0.8
Kaneko et al., 1996[71]	14 FAS	14 Controls	Physiological neural response: auditory event potentials	FAS	78.6	42.9	57.9	66.7	60.7	
		14 Down syndrome	Combination of P300 variables		78.6	85.7	84.6	80	82.1	
Tseng et al., 2013[78]	13 FASD	21 ADHD	19 Features of saccadic eye movements	FASD					90.4	
		18 Controls							79.2	
		21 ADHD and 18 controls			73	91			77.3	

Appendix 3. Predictive Characteristics* of Other Screening Tools for Fetal Alcohol Spectrum Disorders and Its Associated Symptoms (Continued)

Author	PAE/FASD Sample	Comparison Sample	Predictor	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
Zhang et al., 2019[77]	91 FASD	116 Controls	Physiological neural response: eye tracking features of eye tracking, DTI, and neurobehavioral testing	FASD	81.8	87.5			84.8	
			Prosaccade						69.6	
			Antisaccade						76.1	
			Mesasaccade						65.2	
			DTI (4 features)						67.4	
			Neurobehavioral (3 domains)						78.3	
Bookstein et al., 2007[79]	23 PAE	21 Unexposed or lightly exposed	Neuroimage: MRI "hook" feature of corpus callosum	AE	52.2	95.2	92.3	64.5	72.7	
Little & Beaulieu, 2020[80]	79 FASD	81 Controls	Neuroimage: MRI 10 heavily weighted brain regions	FASD	64	88			77	
Burd et al., 1999[84]	1,013 Screened in school system with 6 FAS	1,007 Screened in school/ no FAS diagnosis	Screening tool completed by trained staff > 20	Screened + FASD	100	94.1	9.2	100	94	
Burd et al., 2003[85]	152 FAS	IOM FAS cohort	FAS Diagnostic Checklist-total	FAS	84.9	82.4	75.4	89.5		
	157 pFAS			pFAS	54.3	83.3	66.1	74.1		
	87 PAE not FAS			PAE Not FAS	77	90.8	70.5	93.2		
Grant et al., 2013[86]	25 FASD (FAS, ARND, FAE, static encephalopathy)	463 No PAE	Self-report interview life history screen/addiction severity index	FASD	80.8	65.5			67.6	
Klug et al., 2021[81]	76 FASD	76 Controls	Caregiver Questionnaire: Checklist ARND-BC Parent All Questions	FASD	91.9	95.8	95.2	92.5		
			ARND-BC parent questions positive from binary regression		90.8	92.8	92.4	91.3		
			ARND-BC parent questions positive from continuous regression		89.7	94.5	94	90.5		
			ARND-BC parent questions sum of positive domains		89.7	94.5	94	90.5		

Appendix 3. Predictive Characteristics* of Other Screening Tools for Fetal Alcohol Spectrum Disorders and Its Associated Symptoms (Continued)

Author	PAE/FASD Sample	Comparison Sample	Predictor	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
Nash et al., 2006[82]	54 FASD (ages 6–16)	30 Controls	Caregiver Responses to CBCL (7 items)	FASD				96.5		
		30 Controls	Caregiver responses to CBCL (6/7 items)		86	82				
		30 Controls	Caregiver responses to CBCL (5/7 items)		80	70	80	90.1		
		30 ADHD	Caregiver responses to CBCL (6/7 items)					86.3		
		30 ADHD	Caregiver responses to CBCL (3 items)		81	72				
		30 ADHD	Caregiver responses to CBCL (2 items)		70	80		84.9		
Nguyen et al., 2014[83]	79 PAE + ADHD; 36 PAE – ADHD	90 Controls + ADHD; 16 Controls – ADHD	Caregiver responses to BRIEF	AE					71.4	
Bernes et al., 2021[90]	177 Alcohol exposed CIFASD II	204 Controls CIFASD II	Low cutoff > 1.5 neurobehavioral battery & dysmorphology exam	AE	76.9	76.5	66	84.8	76.6	
			High cutoff > 2 neurobehavioral battery & dysmorphology exam	AE	63.6	87.8	75.5	80.3	78.8	
	177 Alcohol exposed CIFASD III	346 Controls CIFASD III	Low cutoff > 1.5 neurobehavioral battery & dysmorphology exam	AE	83.1	59	50.9	87.2	67.1	
			High cutoff > 2 neurobehavioral battery & dysmorphology exam	AE	66.1	77.5	60	81.7	73.6	
			Classification 3 AE vs. ADHD; latent profile analysis of complex neurobehavioral battery, dysmorphology, growth	AE	59.8	75.8	87.4	40	64	
Coles et al., 2020[91]	82 High risk ARND CoFASP sample of 1st graders	80 No risk CoFASP sample of 1st graders	Comprehensive neuropsychology battery	ARND CoFASP	74.4	83.8	82.4	76	79	

Appendix 3. Predictive Characteristics* of Other Screening Tools for Fetal Alcohol Spectrum Disorders and Its Associated Symptoms (Continued)

Author	PAE/FASD Sample	Comparison Sample	Predictor	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
	85 Low-risk ARND CoFASP sample of 1st graders	73 No risk CoFASP sample of 1st graders			90.6	89	90.6	89	89.9	
Luca et al., 2016[87]	21 FAS	86 No FASD	Neurodevelopmental: quick screen neurological test-2	FAS	31.8	86.1	36.8	83.2	75	
	60 PAE	42 No PAE		AE	18.3	87.5	64.7	46.2	49.1	
Goh et al., 2016[88]	146 CIFASD II AE	288 No AE	Neurodevelopmental: complex neuropsychological battery	AE	74.2	89.9	78.6	87.4	84.6	
	55 CIFASD III child AE	110 No AE	Psychologist decision tree incorporating complex neurobehavioral battery & physical exam	AE	70.7	93.5	87.9	82.9	84.5	
	98 CIFASD III adolescent AE	191 No AE		AE	79.3	87.6	77.4	88.7	84.7	
	146 CIFASD II AE	288 No AE		AE	79.2	80.6	70.7	86.7	80.1	
	55 CIFASD III child AE	110 No AE		AE	63.8	93.4	85.7	80.7	82.1	
	98 CIFASD III adolescent AE	191 No AE		AE	81.3	78.3	71.4	86.2	79.5	
Johnston et al., 2019[92]	43 FASD	20 PAE but no FASD	Movement battery of tests (-2 SD, < 2nd percentile)	FASD	2–38	80–100				
			Movement battery of tests 5th percentile		9–75	68–100				
			Movement battery of tests 9th percentile		35–85	60–85				
			Movement battery of tests 16th percentile		44–83	45–70				
Mattson et al., 2010[93]	CIFASD children ages 8–17; 41 AE/FAS	46 CIFASD controls	Latent profile analysis of a complex battery	AE/FASD	87.8	95.7	94.7	89.8	92	
	CIFASD children ages 8–17; 41 alcohol-exposed/deferred not FAS	60 Controls	Latent profile analysis of a complex battery	AE/deferred FAS	68.4	95	89.7	82.6	84.7	

Appendix 3. Predictive Characteristics* of Other Screening Tools for Fetal Alcohol Spectrum Disorders and Its Associated Symptoms (Continued)

Author	PAE/FASD Sample	Comparison Sample	Predictor	Outcome	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC from ROC
Mattson et al., 2013[89]	CIFASD children (ages 8–17) 209 AE (79 were FAS)	185 Controls; 74 ADHD	Neurodevelopmental: complex neuropsychological battery Latent profile analysis of complex neurobehavioral battery Dysmorphia, growth	AE/FASD	77.2	75.7	57.6	88.6	76.1	
			Classification 2 AE/non-FAS vs. controls	AE/FASD	70.1	72.4	61.7	79.3	71.5	
Thorne & Coggins, 2008;[94] Thorne et al., 2007[95]	16 FASD	16 Normal controls	Narrative speech samples (NSS)-ANRTW	FASD	87.5	75			81.3	0.86
			NSS-PR		81.3	62.5			71.9	0.77
			NSS-ANR		81.3	81.3			81.3	0.76
			NSS-ANR Cutoff 1.7%						100	1
			NSS-AR							0.76
			NSS-Nominal Reference Errors (rNRE)						88	0.9
			NSS-Nominal Reference Errors (rNRE) 2%						97	0.98

*Prediction characteristics evaluated in each study included sensitivity, specificity, NPV, PPV, accuracy, and AUC derived from ROC curves. Sensitivity refers to the probability that the test is positive when the condition is present. Specificity refers to the probability that the test is negative when the condition is not present. PPV refers to the probability that the condition, is present when the test is positive. NPV refers to the probability that the condition is not present when the test is negative. Accuracy refers to the overall probability that the case is correctly classified from the test. Finally, AUC is derived from creating receiver operating curves by plotting the true positive rate (sensitivity) relative to the false positive rate (1-specificity). The AUC references the area on the graph created by the regression line relative to the chance rate of prediction. Values of 1 would indicate perfect condition, and values of 0.50 would indicate chance prediction using a yes/no model. Predictive validity values are presented as percentages with the exception of AUC values, which are reported in proportions of accurate diagnostic classification with values of 0 to 1.00. The different categories of predictive data (facial, neurophysiological, neuroimaging, questionnaire, and psychological performance measures are shaded from white to dark blue.

Note: 2D, two-dimensional; 3D, three-dimensional; ADHD, attention-deficit/hyperactivity disorder; AE, alcohol-exposed; ANRTW, Ambiguous Normal Reference Total Word; ARND, alcohol-related neurodevelopmental disorder; ARND-BC, Alcohol-Related Neurodevelopmental Disorder Behavior Checklist; AUC, area under the curve; AUD, alcohol use disorder; BRIEF, Behavior Rating Inventory of Executive Function; BSI-FASD, biographic screening interview for fetal alcohol spectrum disorders; CBCL, Child Behavior Checklist; CIFASD, Collaborative Initiative on Fetal Alcohol Spectrum Disorders; CIFASD-II, CIFASD, Phase II; CIFASD-III, CIFASD, Phase III; CoFASP, Collaboration on FASD Prevalence; COR, cardiac orienting response; DTI, diffusion tensor imaging; FAE, fetal alcohol effect; FAS, fetal alcohol syndrome; FASD, fetal alcohol syndrome disorders; IOM, Institute of Medicine; MRI, magnetic resonance imaging; NPV, negative predictive validity; NSS, narrative speech samples; NSS-ANR, narrative speech sample–ambiguous normal reference; NSS-AR, narrative speech sample–ambiguity rate; NSS-PR, narrative speech sample–pronoun reference; OUD, opioid use disorder; PAE, prenatal alcohol exposure; pFAS, partial fetal alcohol syndrome; PPV, positive predictive value; rNRE, rate of nominal reference errors; ROC, receiver operating characteristic; SD, standard deviation.

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Reducing Prenatal Alcohol Exposure and the Incidence of FASD: Is the Past Prologue?

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PURPOSE: This narrative review summarizes and synthesizes the clinical trials and randomized clinical trials that evaluated selected and targeted approaches to reducing preconception and prenatal alcohol exposure (PAE) and alcohol-exposed pregnancy (AEP) since 2011.
SEARCH METHODS: A professional hospital librarian completed the primary search using strategies specified within this review, resulting in 94 records returned in PubMed, Ovid MEDLINE, Clinical Key, the World Health Organization International Clinical Trials Registry Platform, and ClinicalTrials.gov. The author completed two supplementary literature searches.
SEARCH RESULTS: From the total of 238 records returned from the three searches, 217 records were eliminated. Elimination reasons included other medical problem (119); duplicate entry (34); no content/results (23); secondary analysis (16); focus on effects of PAE (9); treatment of childhood fetal alcohol spectrum disorders (FASD) (6); maternal risk factors (3); and other (7). The remaining 21 studies were included with four overarching themes: (1) case management efforts (n = 4); (2) preconception efforts to reduce AEP (n = 5); (3) motivational interviewing and screening, brief intervention, and referral to treatment (n = 2); and (4) use of technology to deliver the intervention (n = 10).
DISCUSSION AND CONCLUSIONS: Case management and home visits did not appear to have strong current empirical support. Study limitations included small sample sizes and no comparison groups, whereas larger efforts did not demonstrate definitive advantages to justify this intensive approach. The studies of preconception efforts, all based on the Project CHOICES approach, had similar outcomes, with the reduction in AEP risk largely due to improved contraception in women of childbearing age who were sexually active and drank alcohol but were not pregnant. It is unknown whether these women refrained from alcohol use when they became pregnant. Two studies of motivational interviewing to reduce prenatal alcohol use did not demonstrate the efficacy of the intervention. Both were small, with less than 200 pregnant women combined; moreover, the study samples had low baseline levels of alcohol use, allowing little opportunity for improvement. Finally, studies evaluating the impact of technological approaches to reducing AEP were reviewed. These exploratory investigations had small sample sizes and provided preliminary evaluations of techniques such as text messages, telephone contact, computer-based screening, and motivational interviewing. The potentially promising findings may inform future research and clinical efforts. Future directions may include research to address the limitations of the evidence to date and should reflect the complexities of FASD that include the biological and social context associated with prenatal alcohol use.

KEYWORDS: alcohol; prevention; fetal alcohol effects; fetal alcohol syndromes; preconception care; fetal alcohol spectrum disorders

Prenatal alcohol exposure (PAE) is linked to miscarriage, stillbirth, preterm birth, sudden infant death syndrome, and fetal alcohol spectrum disorders (FASD).[1] Although PAE is the sole necessary cause of FASD, the etiology of this leading preventable cause of disability is multifaceted and complex, including lifestyle, maternal, sociodemographic, social, gestational, and genetic risk factors.[2-4] As the identification of specific maternal drinking behaviors related to FASD remains inconclusive, efforts to reduce PAE and the incidence of FASD continue to be necessary.[5,6]

The last issue of *Alcohol Research: Current Reviews* dedicated to FASD was published in 2011, when the journal was named *Alcohol Research & Health*. At that time, maternal risk factors for FASD were recognized to be multidimensional and included quantity, frequency, and timing of alcohol exposure; maternal age; and social relationships, among others. Because it was not known then which factors were most likely to lead to having children with FASD,[7] prevention efforts were important. Universal approaches—such as broad media campaigns and warning labels on alcohol beverage containers—were not particularly effective in reducing alcohol use during pregnancy. Limited research was available on selected and targeted prevention efforts aimed at women in special risk groups or at women known to be more vulnerable because of binge drinking. However, screening instruments to identify women at risk of prenatal alcohol use and administration of brief interventions in the clinic had positive effects in reducing drinking during pregnancy.[8]

In 2014, the World Health Organization (WHO) published *Guidelines for the Identification and Management of Substance Use and Substance Use Disorders in Pregnancy*.[9] The Guidelines were developed to enable professionals to assist women who were pregnant and who used alcohol or other drugs to optimize healthy outcomes for their patients and the fetus or infant. The Guidelines reflected the collaboration of the WHO internal steering group, Guideline Development Group, and External Review Group. Based on the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) system for assessing quality of evidence, the Guidelines focused on six areas, two of which are relevant to this review: (1) screening and brief intervention (SBI), and (2) psychosocial interventions to prevent PAE and the incidence of FASD.

Because much of the evidence supporting the effectiveness of SBI predated the GRADE standards, the WHO Guidelines had "strong" recommendations for SBI despite "low" quality of evidence. SBI was supported because the potential benefits outweighed the potential harms. On the other hand, psychosocial interventions for substance use disorders in pregnancy received only a "conditional" recommendation because of "very low" evidence to support their use. Examples of psychosocial interventions include motivational interviewing and home visits following delivery to support abstinence.

The WHO recommendations are reflected in the 2015 *Committee Opinion* of the American College of Obstetricians and Gynecologists,[10] which provided an ethical framework to encourage physicians to use routine screening, brief intervention, and referral to treatment (SBIRT) for alcohol and other substance use among their obstetrics/gynecology patients as appropriate. Similarly, the Society of Obstetricians and Gynaecologists of Canada published a clinical practice guideline in 2020, which recommended that all pregnant women be asked about alcohol consumption using evidence-based SBI approaches.[11]

While the most prudent recommendation continues to be abstinence from alcohol throughout pregnancy, some pregnant women continue to drink alcohol.[12,13] Past 30-day reports of alcohol use by pregnant women between 2018 and 2020 indicated that 14% reported current drinking (i.e., at least one drink in the past 30 days) and 5% reported binge drinking (i.e., four or more drinks in one sitting, at least once in the past 30 days).[14] Both measures were 2 percentage points higher than the 2015–2017 rates, but these changes were not statistically significant.[14]

Moreover, some research has suggested that reports about past 30-day alcohol use by pregnant women underestimate the true extent of prenatal alcohol use. Among 4,088 randomly selected control mothers in the National Birth Defects Prevention Study, 30% had "some alcohol" while pregnant and 8% reported binge drinking (defined as four or more alcoholic drinks per occasion).[15] The Generation R Study, a population-based prospective cohort study from fetal life to adulthood of 7,141 individuals in the Netherlands, found that 37% of pregnant women continued to consume alcohol after pregnancy was known.[16]

These rates of alcohol use during pregnancy stand in contrast to the Healthy People 2030 goals established by the U.S. Department of Health and Human Services. Healthy People 2030 sets data-driven national objectives to improve health and well-being over the next decade, including the goal of increasing abstinence from alcohol among pregnant women to 92%.[2]

This narrative review focuses on the clinical trials and randomized clinical trials that have evaluated selected and targeted approaches to reducing preconception alcohol exposure, PAE, and AEP since 2011, when *Alcohol Research: Current Reviews* (then called *Alcohol Research & Health*) last focused on this area. Examples of these approaches include case management for pregnant women at risk of drinking; applications of the Project CHOICES preconception approach to reduce AEP by adoption of effective contraception in clinical and other settings; motivational interviewing among pregnant women; and utilization of technology-based interventions such as the telephone, Internet, and text messaging to

reduce prenatal alcohol exposure. The focus on clinical and psychosocial efforts was chosen because these approaches are still needed to reduce AEP and the prevalence of FASD, to establish a new baseline for inquiry, and to encourage innovations moving forward.

Search Methods

A literature search was conducted in November 2021 of the PubMed, Ovid MEDLINE, Clinical Key, WHO International Clinical Trials Registry Platform (ICTRP), and ClinicalTrials.gov databases using these search strategies:

- For PubMed: ("alcohol drinking"[MeSH Major Topic] OR prenatal alcohol[Title/Abstract]) AND ("Pregnancy"[MeSH Terms] OR "Prenatal Care"[MeSH Terms]) AND ("clinical trial"[Publication Type] OR "clinical trial, phase I"[Publication Type] OR "clinical trial, phase II"[Publication Type] OR "clinical trial, phase III"[Publication Type] OR "clinical trial, phase IV"[Publication Type] OR "controlled clinical trial"[Publication Type] OR "pragmatic clinical trial"[Publication Type] OR "randomized controlled trial"[Publication Type]);
- For Ovid MEDLINE: (prenatal* adj5 (alcohol or ethanol)).tw. limited to (clinical trial, phase I or clinical trial, phase II or clinical trial, phase III or clinical trial, phase IV or clinical trial or controlled clinical trial or pragmatic clinical trial or randomized controlled trial);
- For Clinical Key: "prenatal alcohol" filtered by Clinical Trials;
- For WHO ICTRP and ClinicalTrials.gov: "prenatal alcohol."

The search was limited to articles published in English over the last 10 years. A professional librarian (see Acknowledgments) at the author's hospital executed these foundational literature searches resulting in 94 hits.

In addition, the author completed two supplementary literature searches using PubMed and the search terms—(1) prevention and fetal alcohol spectrum disorders (FASD), and (2) PCAP (parent child assistance program), which resulted in 19 and 125 hits, respectively. These additional literature searches were undertaken to include prevention of FASD and the parent-child assistance program studies that were not returned by the other searches but are a case management approach in the United States well described in the decade before 2011.[17]

Table 1 summarizes the literature tracking.[18,19] Of the total of 238 records returned by the three searches, 217 records were eliminated. Elimination reasons included: other medical problem (119), duplicate entry (34), no content/results (23), secondary analysis to allow consistent focus on primary investigations (16), focus on PAE effects (9), treatment of childhood FASD (6), maternal risk factors (3), and other (7). A total of 21 studies were thus included.

Results

Table 2 summarizes the 21 studies selected for review. They are sorted into four overarching themes: (1) case management efforts, (2) preconception trials to reduce AEP, (3) motivational interviewing and SBIRT, and (4) use of technology.

Effectiveness of Case Management Efforts

Several clinical trials evaluated case management or home visits as an intervention to reduce prenatal alcohol use. Most relied on motivational interviewing and community reinforcement. Two were unblinded, indicated prevention efforts without comparison groups,[20,21] whereas the other two used a randomized controlled trial design.[22,23]

May et al. reported on a sample of 41 women from the Cape provinces of South Africa who were deemed to be at high risk for bearing a child with FASD; 88% of the women were pregnant at intake.[20] Women were considered at high risk if they (1) had already borne one child with FASD or had drank heavily during a prior pregnancy; (2) were currently drinking eight or more drinks per week or one binge of three or more drinks per day; or (3) had a "high score" on the self-administered questionnaire (score > 2) or the Alcohol Use Disorders Identification Test (AUDIT) (score > 8). They were offered 18 months of case management (CM) with data collected at baseline and at 6, 12, and 18 months after starting CM; there was 27% loss to follow-up before 18 months. The AUDIT was used to measure drinking during the study. For the 29 women involved in CM for the entire 18 months of the study, the mean AUDIT score was 19.4 (*SD* = 6.7) at baseline, dropped to 9.7 at 6 months, rose to 10.8 at 12 months, and 12.3 at 18 months. Limitations include the indirect measure of alcohol use, high attrition rate, lack of a comparison group, and small sample size. It also was unclear whether the case management strategies are feasible with regards to resources in other settings (e.g., clinical rather than research).

De Vries et al. conducted a similar prospective intervention study at community health clinics in the Western Cape province of South Africa between January 2009 and June 2011.[21] The researchers offered CM to support pregnant women who drink heavily to achieve abstinence or reduction in alcohol during pregnancy and in the first postpartum year. These investigators used the same definition of "heavy drinking" and risk of having a child with FASD.[20] CM incorporated life management, motivational interviewing techniques, and the community reinforcement approach. A total of 67 women enrolled, most during their second trimester and all at high risk for bearing a child with FASD; loss to follow-up at 18 months was 24% loss. The researchers found that compared with baseline, alcohol use decreased from the time of enrollment to the first 6 months of

CM, but increased at 12 and 18 months. Limitations were similar to those mentioned for the study by May et al.[20]

Rotheram-Borus et al. examined the effect of a one-time home visiting intervention on prenatal alcohol use and problematic drinking, as well as the association of home visiting and alcohol use on children's behavioral, cognitive, and health outcomes among 1,236 mothers and their children from pregnancy to 5 years afterwards.[22] The study, which

Table 1: Literature Search Tracking Summary

Search #	Date of Search	Database	Search Terms	Number of Hits	Excluded
1	November 2021	PubMed	("alcohol drinking"[MeSH Major Topic] OR prenatal alcohol[Title/Abstract]) AND ("Pregnancy"[MeSH Terms] OR "Prenatal Care"[MeSH Terms]) AND ("clinical trial"[Publication Type] OR "clinical trial, phase I"[Publication Type] OR "clinical trial, phase II"[Publication Type] OR "clinical trial, phase III"[Publication Type] OR "clinical trial, phase IV"[Publication Type] OR "controlled clinical trial"[Publication Type] OR "pragmatic clinical trial"[Publication Type] OR "randomized controlled trial"[Publication Type])	94	20 duplicates 20 no abstract/content 12 secondary analysis 6 treatment of children with FASD 6 PAE effects 3 protocol only 3 maternal factors 1 prevalence 1 biomarker 1 cross-sectional 1 postpartum 1 universal
		Ovid MEDLINE	(prenatal* adj5 (alcohol or ethanol)).tw. limited to (clinical trial, phase I or clinical trial, phase II or clinical trial, phase III or clinical trial, phase IV or clinical trial or controlled clinical trial or pragmatic clinical trial or randomized controlled trial)		
		Clinical Key	"prenatal alcohol" filtered by Clinical Trials		
		WHO ICTRP and ClinicalTrials.gov	"prenatal alcohol"		
			Search limited to articles published in English over the last 10 years.		**Yield: 19 included**
2	May 2022	PubMed	prevention AND fetal alcohol spectrum disorders	19	13 duplicates of Search 1 4 outside review scope (3 on FASD effects, 1 on beliefs)
			Search limited to articles on randomized clinical trials and clinical trials published in English over the past 10 years.		**Yield: 2 included**
3	May 2022	PubMed	PCAP (parent child assistance program)	125	119 acronyms for other medical entities 4 secondary analyses 1 duplicate 1 qualitative study
			Search limited to articles published in English over the past 10 years.		**Yield: 0 included**

Note: FASD, fetal alcohol spectrum disorders; MeSH, Medical Subject Headings; PAE, prenatal alcohol exposure; PCAP, parent-child assistance program; WHO ICTRP, World Health Organization International Clinical Trials Registry Platform.

was conducted in Cape Town, South Africa, included 1,236 mothers and their children who participated in a program that allowed up to 5 years of contact. The study compared standard care and the intervention, known as the Philani Program, using a longitudinal cluster-randomized design. The Philani Program trained township women as Mentor Mothers who offered a brief, one-visit intervention on alcohol prevention in pregnancy, in addition to their other home-based primary-care efforts, such as rehabilitation of underweight children or prevention of mother-to-child HIV transmission. At baseline, 10% of the pregnant women in both conditions drank alcohol, and both groups also showed reductions in alcohol use over the course of pregnancy and increases in alcohol use afterwards. There were no statistically significant differences in alcohol use between the groups at any time with this brief, one-time intervention.

More recently, Catherine et al. reported findings from the Nurse-Family Partnership (NFP), a program in which public health nurses provided frequent home visits from early pregnancy until the children were 2 years old.[23] With a focus on first-time parents experiencing socioeconomic disadvantage, the analysis included 739 pregnant girls and women ages 14 to 24 who were randomly allocated 1:1 to the intervention (NFP plus treatment as usual) or control (treatment as usual). NFP guidelines allowed as many as 14 prenatal visits and 50 postpartum visits, during which nurses helped participants to identify and meet health and social goals, including reducing prenatal substance use. Changes in the use of nicotine cigarettes and alcohol use by 34–36 weeks' gestation were prespecified prenatal secondary outcomes. The research team found no evidence that NFP was effective in reducing rates of prenatal use of cigarettes or alcohol; the intervention condition was associated with reduced cannabis use.

In conclusion, the few published studies of CM approaches to reducing prenatal alcohol use did not provide strong empirical support for the effectiveness of these measures. These findings are consistent with the assessment published in the WHO Guidelines.[9]

Effectiveness of Preconception Measures to Prevent AEP

Four randomized trials and one clinical trial of efforts to reduce AEP, some of which were based on the CHOICES approach,[24] were published in the past decade. These efforts used motivational interviewing and cognitive behavior strategies and targeted the adoption of effective contraception and reduction of alcohol use.

Rendall-Mkosi et al. randomized 165 nonpregnant women who were considered to be at risk for AEP to either a five-session motivational interviewing intervention or a control condition.[25] Women were classified as being at risk if they (1) were ages 18 to 44, (2) were not pregnant, (3) had engaged in risky drinking in the past 3 months (defined as more than five drinks per episode or more than seven drinks per week), (4) had used ineffective or no contraception, (5) were able to conceive, (6) had vaginal sex in the past 3 months, and (7) lived within a certain distance from the main town. The study originally included a third arm with a group-based life-skills training intervention, which was terminated because of difficulties with implementation. Although modeled on Project CHOICES, this study used simplified data collection tools at baseline and at 3 and 12 months afterward. The main finding was that women in the motivational interviewing group were more than twice as likely as women in the control group to lower their risk for AEP at 12-month follow-up (*OR* = 2.64, 95% *CI* [1.18, 5.94]); this difference was reduced but remained significant using an intention-to-treat analysis (*OR* = 2.19, 95% *CI* [1.05, 4.65]). However, the reduction in risk for AEP was due mainly to the improved use of contraceptives rather than a reduction in alcohol use.

Ingersoll et al. tested a one-session motivational AEP prevention intervention among 217 women who had at least one episode of unprotected vaginal sex with a male partner and drank at risky levels (defined as more than three drinks per occasion or more than seven drinks per week) in the past 90 days.[26] The women completed baseline assessments and were randomized to motivational interviewing plus assessment feedback (EARLY), informational video, or informational brochure conditions. Outcomes were drinks per drinking day, ineffective contraception rate, and AEP risk at 3 and 6 months. Results showed reductions in drinking, increased contraception, and reduced AEP risk for all conditions. Because all conditions included an assessment of baseline drinking behaviors, it appears that raising risk awareness through assessment could be impactful. The effect from assessment on subsequent alcohol use is supported by other studies.[27]

Sobell et al. conducted a CHOICES-like randomized controlled study for 354 women at risk for AEP; participants included 145 college students.[28] Risk for AEP was defined as being of childbearing age (18-45 years old), having had heterosexual vaginal intercourse with ineffective contraception, and having consumed eight or more standard drinks per week or five or more standard drinks per episode. (Unless otherwise indicated, a standard drink is defined as containing 14 g of alcohol and corresponds to 12 oz of beer [5% alcohol], 5 oz of wine [12% alcohol], or 1.5 oz of liquor or spirits [40% alcohol].) The women were randomized either to receive motivational feedback based on Project CHOICES or to a control group receiving information

only. Similar to the findings by Ingersoll et al.,[26] there was no significant difference between the two interventions at 6-month follow-up. For all groups, risk reduction occurred primarily through increasing effective contraception.

Velasquez et al. tested the efficacy of CHOICES Plus, a preconception intervention for reducing the risk of AEP and tobacco-exposed pregnancy, among 261 nonpregnant women of childbearing age (18 to 44 years) attending primary care clinics in a large Texas public health system.[29] The women were sexually active, with at least one episode of unprotected vaginal sex with a male partner, and were considered to show risky drinking (defined as more than three drinks per episode or more than seven drinks per week). In this study, women were randomized to either two CHOICES Plus sessions and a contraceptive visit or to brief advice and referral to community resources. In an intention-to-treat analysis at 9-month follow-up, the Project CHOICES Plus group was more likely than the brief-advice group to reduce the risk of AEP, with an incidence rate of 0.620 (95% *CI* [0.511, 0.757]) and an absolute risk reduction of −0.233 (95% *CI* [−0.239, −0.226]). The Project CHOICES group at risk for both exposures was also more likely to reduce risk of tobacco-exposed pregnancy (incidence rate ratio, 0.597; 95% *CI* [0.424, 0.840]), and absolute risk reduction of −0.233 (95% *CI* [−0.019, −0.521]).

Hanson et al. tailored the Project CHOICES approach for a prevention program run by the Oglala Sioux Tribe so that the program was culturally appropriate for American Indian women.[30] The program's effectiveness was examined in two communities on the reservation and one community off the reservation. A total of 193 nonpregnant Native American women were enrolled, and 51% completed baseline assessment and both 3- and 6-month follow-up; there was no comparison group. The participants were considered at risk for AEP because they exceeded low-risk drinking limits for women (defined as four or more drinks per occasion or eight or more drinks per week) and were not using effective contraception while sexually active. Results were consistent with the findings of other CHOICES-related research; thus, women receiving the Oglala Sioux Tribe CHOICES intervention were significantly more likely to improve birth control (68% at 3 months and 62% at 6 months) than to drink less (10% and 20% reductions in binge drinking at 3 and 6 months, respectively).

In conclusion, the preconception Project CHOICES approach to preventing AEP appears to exert its effect primarily through improving effective contraception in women of childbearing age who are sexually active, rather than through reducing alcohol use. Strengths of the studies assessing the CHOICES approach include the use of consistent approaches to the intervention and, in most cases, use of comparison groups.

Effectiveness of Motivational Interviewing and SBIRT

In the past decade, two studies assessed the effectiveness of motivational enhancement approaches based on screening and brief intervention in decreasing prenatal alcohol use.

Osterman et al. examined the usefulness of a single-session motivational interviewing intervention in decreasing alcohol use during pregnancy.[31] The intervention employed theory-based mechanisms of behavior change as guided by the self-determination theory. The study included 67 pregnant women who reported past year alcohol use, 59 of whom were randomized either to the intervention or to a comparison group; all participants completed baseline and follow-up interviews. The intervention was not found to be effective in decreasing prenatal drinking behaviors. In a second study, the same research team tested the effectiveness of a single-session motivational interview to decrease alcohol use during pregnancy.[32] This study included 122 pregnant women who drank in the past year who were randomized to either the intervention or a comparison group. Treatment effects over time were evaluated with Poisson and linear regression with generalized estimating equation. Again, motivational interviewing was not found effective in decreasing prenatal alcohol use. The investigators suggested that the low levels of baseline alcohol use in the study sample left little room for improvement. A secondary analysis by the same research group is beyond the scope of this review.[33]

These two studies, both of which were limited by small participant numbers, indicate that the promise of motivational interviewing or screening and brief intervention in reducing prenatal alcohol use cannot be confirmed. The two trials do not demonstrate that a single-session intervention will influence behavior over the course of a pregnancy.

Effectiveness of Technology-Based Interventions

Several trials evaluated the impact of text messages, brief intervention delivered over the telephone, computer-delivered screening and brief intervention, and an Internet intervention delivered preconception on risk of AEP. Additionally, one study assessed a novel application of four-dimensional (4D) ultrasound of fetal development as an intervention.

Text messages

Evans et al. conducted two pilot studies of the mobile health program Text4baby in two groups of women. One group included 123 women with low income seeking prenatal care;[34] the other included 943 pregnant military women presenting for care at a military medical center.[35] Text4baby is a mobile health program based on social cognitive theory in which health messages are delivered to pregnant women and new mothers to improve their

health care beliefs and behaviors with the goal of improving health status and clinical outcomes. In both studies, the women completed a baseline assessment survey before being randomized to the intervention group (Text4baby plus usual care or usual care alone). Follow-up was planned at approximately 28 weeks' gestation for the low-income group and 4 weeks after enrollment for the military group.

In the study of women with low income, 73% participated at follow-up. The Text4baby intervention was significantly associated with increased agreement with the statement, "I am prepared to be a new mother," between baseline and follow-up (*OR* = 2.73, 95% *CI* [1.04, 7.18], *p* = .042). Furthermore, among mothers of low income with a high school education or higher, the intervention was significantly associated with increased agreement with attitudes against alcohol consumption during pregnancy (*OR* = 2.80, 95% *CI* [1.13, 6.90], *p* = .026).[34]

The study involving military women had greater loss to follow-up, with only 49% of participants completing the assessment at 4-week follow-up. Moreover, the results were less encouraging. In a generalized estimating equations logistic regression model adjusted for four socioeconomic variables, imputations for missing values for marital status and race, and inverse probability weighting to account for attrition, there were marginally significant effects for improved strong agreement with the statements, "If I visit my health care provider on a regular basis, I will be a healthy new mother" as well as "Drinking alcohol will harm the health of my developing baby." The Text4baby intervention had no effects on any of the measured behaviors (e.g., alcohol use).

Telephone-based interventions

Two studies evaluated a telephone-based preconception intervention for women of childbearing age who were sexually active, did not use effective contraception, and consumed alcohol at "risky levels." Farrell-Carnahan et al. offered a one-session, remote-delivered, preconception, motivational interviewing-based AEP intervention (EARLY Remote) to 46 non–treatment-seeking community women;[36] there was no comparison group. The participants were women who were sexually active and consumed seven or more standard drinks per week and/or three or more standard drinks per drinking episode in the past 90 days and who did not use reliable contraception. All participants received the baseline, 3-month, and 6-month assessments via telephone. Both the number of drinks per drinking day and the rate of unreliable contraception decreased over time.

The study by Wilton et al. included 132 women ages 18 to 44 who screened positive for drinking at risky levels (defined as more than seven drinks per week or more than three drinks in any one day) and who were not using effective contraception.[37] After completing a baseline assessment interview, the women were randomized to a brief two-session intervention delivered either via telephone or in person. There was no significant difference between the two groups in the success of the brief intervention at 6-month follow-up. Overall, participants demonstrated small reductions in alcohol use (11%) and larger increases in the effective use of contraceptives. The intervention modality was not a significant predictor of any outcomes after controlling for potential confounding measures.

Computer-based interventions

Several computer-based interventions have been evaluated in preconception and pregnant women. Van der Wulp et al. completed a cluster randomized trial in which 60 Dutch midwifery practices were randomly assigned to one of three conditions: health counseling, computer-tailored feedback, and usual care.[38] Participating women needed to understand Dutch, be age 18 or older, be no more than 12 weeks pregnant, and have consumed alcohol while pregnant. Among the participants, 135 women received counseling from their nurse-midwife according to a health-counseling protocol that included seven steps in three feedback sessions, 116 women received usual care plus three computer-tailored feedback letters via the Internet, and 142 women received usual care or routine alcohol care from their nurse-midwives. The effect of the interventions on alcohol use was assessed at 3 or 6 months.

Results from this trial were promising overall because after 6 months and three feedback letters, the participants receiving computer-tailored feedback stopped using alcohol more often than did those receiving usual care (53/68 women or 78% versus 51/93 women or 55%, respectively; *p* = .04). Limitations to the generalizability of findings include the lack of statistical power and suboptimal implementation of the health counseling intervention; consequently, no effect size was published.

Ondersma et al. conducted a pilot study with 48 pregnant women at an urban prenatal care clinic who screened positive for alcohol risk—defined as scoring positive on the T-ACE (tolerance, annoyed, cut-down, eye-opener) questionnaire, as well as drinking weekly or more in the past month, or having four or more drinks at least once a month in the 12 months before becoming pregnant.[39] Participants were randomized to either electronic screening and brief intervention (e-SBI) or a control session on infant nutrition. The e-SBI was a 20-minute interactive session based on motivational interviewing and self-determination theory, followed by three tailored mailings to participants. The follow-up assessment occurred in person after childbirth and before hospital discharge; it was blinded to treatment condition. Results for alcohol use and birth outcomes were of moderate size and favored the intervention (*OR* = 3.4, *p* = .19 and *OR* = 3.3, *p* = .09, respectively). Although

this pilot study showed that the technology-delivered intervention was feasible and acceptable, it could not evaluate the separate contributions of the e-SBI and the mailings or provide an effect-size.

Montag et al. randomized 263 American Indian/Alaska Native women of childbearing age (including 29 pregnant women and 234 nonpregnant women) to a culturally targeted online SBIRT intervention (eCHECKUP TO GO) or treatment as usual.[40] eCHECKUP TO GO is a web-based brief assessment and intervention. All participants completed a baseline survey that evaluated awareness of FASD, usual alcohol consumption, and demographic background among other factors. With little loss to follow-up (6%), the investigators found that risky drinking behavior (defined as three or more standard drinks per occasion and/or eight or more drinks per week) and risk of AEP were reduced in both the intervention and control groups. There was evidence of a time effect but no statistically significant treatment effect. Study limitations included self-selected volunteers (rather than women who met criteria such as being pregnant and drinking at certain levels) in addition to the usual concerns about self-report.

Ingersoll et al. conducted a pilot randomized trial of an Internet intervention to reduce the risk of AEP among 71 women drinking at a risky level (defined as four or more standard drinks per episode in the past 3 months) without effective or consistent contraception.[41] The women were randomized either to a six-core automated, interactive, and tailored Internet intervention—the Contraception and Alcohol Risk Reduction Internet Intervention (CARRII) based on the CHOICES intervention—or to a static, untailored patient education website offering the same content as CARRII (e.g., information about AEP, FASD, and alcohol use among women). The investigators then assessed the intervention's effect on AEP risk. Of the participants, 64 women completed 6-month follow-up. Women in both conditions reduced risky drinking by less than 20% at 6 months; however, those receiving CARRII demonstrated significant reductions in the proportion of unprotected sex from pretreatment to posttreatment (32%) and to 6-month follow-up (30%).

Wernette et al. conducted a two-group, randomized controlled trial of 50 pregnant women with an average of 13 weeks' gestation attending an inner-city prenatal clinic.[42] Inclusion criteria included pregnant women who endorsed (1) vaginal (or anal) sex without a condom at least once in the past 30 days, (2) unplanned pregnancy, and (3) current alcohol or drug use or at risk for the same because of a positive alcohol (T-ACE) or drug (Substance Use Risk Profile-Pregnancy) screen. The intervention group was given a computer-delivered, single-session brief motivational interview with booster session, both of which addressed substance use and risk of sexually transmitted infection. The attention control condition included answering questions related to television shows and providing subjective ratings. At 4-month follow-up, participants in the intervention arm had a significant reduction in any marijuana or alcohol use compared to the control arm (54% versus 16%, $p = .015$) but an insignificant reduction in vaginal sex without a condom. Potential limitations included reliance on self-report of risk behaviors, inclusion of only English-speaking participants, imbalance in randomization (31 in the intervention group, 19 in the control group), and unblinded research staff.

Use of other technology: 4D ultrasound

Jussila et al. conducted a novel, randomized controlled trial among pregnant women attending an obstetric outpatient clinic in Finland.[43] Ninety women were referred to the clinic because of current or recent substance use—defined as self-reported or documented illicit substance use, abuse of prescription medication or alcohol within the past 3 years or during a previous pregnancy, and/or a score of 3 or higher on the Tolerance-Worry-Eye-Opener-Annoy-Cut-Down (TWEAK) alcohol screening tool. They were randomized either to a control group ($n = 44$) receiving treatment as usual or to an intervention group ($n = 46$) that included interactive use of 4D ultrasound visualization of the fetus at 24, 30, and 34 weeks and a pregnancy diary. The ultrasounds and diary were designed to enhance prenatal parental mentalization and maternal-fetal attachment. With an 89% retention rate overall, retention was significantly higher in the intervention group than in the control group (96% vs. 82%, $p < .05$). Although 74% of the intervention group participated in all three ultrasound sessions, only 59% participated in all scheduled obstetric sessions (compared to 83% of the control group, $p < .02$). Fetal drug exposure (as measured in meconium samples) and perinatal outcomes (i.e., rates of small for gestational age babies) were similar in both groups. This study did not focus on prenatal alcohol use, but included women at general risk of substance use.

In conclusion, the use of technology approaches for offering education and intervention appears to be a potentially promising approach to reducing AEP. However, most studies thus far have been small, reflecting their exploratory and innovative nature.

Table 2: Study Summaries

Case Management

Author	Design	Main Findings/Comments
May et al., 2013[20]	Prospective intervention study of women at high risk of drinking (*n* = 41); 88% pregnant at enrollment. CM with 6-, 12-, and 18-month follow-up.	Although it is difficult to achieve enduring change in drinking in this group, pregnant women did drink less at 6- and 18-month follow-up.
De Vries et al., 2016[21]	Prospective intervention study of pregnant South African women (*n* = 67). Complete data available for 50 of 67 women; no comparison group. CM with drinking measured at baseline and at 6, 12, and 18 months.	Mean reductions in drinking were seen at 6 months, but higher levels were observed at 12 and 18 months over baseline.
Rotheram-Borus et al., 2019[22]	Longitudinal cluster randomized trial of pregnant mothers (*n* = 1,236) from 24 different South African neighborhoods. Severely impoverished sample (e.g., 53% with running water). Standard clinical care vs. CM (Philani Program) with home visits/paraprofessional coaching on drinking from pregnancy to 5 years post-birth.	Women in the Philani Program drank less postnatally, but all women gradually returned to pre-pregnancy rates of drinking.
Catherine et al., 2020[23]	Analysis of prenatal secondary outcomes in an ongoing RCT of pregnant women (*n* = 739). NFP with CM + existing services vs. existing services only. Analyses were intention-to-treat and mixed-effect models for longitudinal and clustered data to estimate intervention effects.	NFP had no effect on reducing rates of prenatal use of cigarettes or alcohol, but did lead to reduced prenatal use of cannabis.

Preconception Trials to Prevent AEP

Author	Design	Main Findings/Comments
Rendall-Mkosi et al., 2013[25]	RCT of MI in women at risk for AEP (*n* = 165). Five-session MI intervention, timed and structured evaluations preintervention and 3 and 12 months afterwards. Outcome was AEP at 12 months, modeled on Project CHOICES. Three conditions: MI, life skills, and control. Life skills stopped after 30 days due to poor adherence.	The MI group was more than twice as likely as the control group to lower their risk for AEP at 12 months (*OR* = 2.64); intention-to-treat analysis reduced the odds ratio to 2.19. Reduction in AEP risk was due mainly to the use of contraception rather than reduced drinking. There was discussion that, compared with other health professionals, medical doctors have greater success in effecting behavior changes.
Ingersoll et al., 2013[26]	RCT to test a one-session MI to reduce AEP in community women (*n* = 217), to either MI + assessment feedback (EARLY), informational video vs. informational brochure.	All interventions associated with drinks per drinking day, ineffective contraception rate, and AEP risk at 3 and 6 months. One-session EARLY intervention had less powerful effects than multiple sessions on AEP.
Sobell et al., 2017[28]	RCT of women at risk for AEP (*n* = 354); 44% minorities. MI is based on CHOICES vs. information only.	No significant difference was seen between the interventions. Comment: "The most effective AEP prevention strategy is to simply communicate to those at risk that they could become pregnant."
Velasquez et al., 2017[29]	Women ages 18–44, not pregnant, not sterile, drinking > 3 drinks per drinking day or > 7 drinks per week, sexually active, not using effective contraception. RCT with two intervention groups: CHOICES Plus (*n* = 131) vs. brief advice (*n* = 130) in 12 primary care clinics in large Texas public health system.	Primary outcomes were reduced risk for AEP and TEP through 9-month follow-up. Intention-to-treat analysis across 9 months and CHOICES Plus significantly reduced risk of AEP and TEP.
Hanson et al., 2015[30]	American Indian women, not pregnant, enrolled in the Ogala Sioux Tribe CHOICES program (*n* = 193) and at risk for AEP.	51% completed 3- and 6-month follow-up. Risk for AEP reduced by improved contraception rather than reduced binge drinking.

Table 2: Study Summaries (cont)

Motivational Interviewing/SBIRT

Author	Design	Main Findings/Comments
Osterman & Dyehouse, 2012[31]	RCT of MI compared to comparison group (*n* = 67); 56 pregnant women completed all study procedures. Self-determination theory applied to increase understanding of the mechanisms of MI. Two group pretest and posttest study of convenience sample. Three basic psychological needs: autonomy, competence, relatedness.	Structural equation modeling determined the direct, indirect, and total effects on the MI intervention on outcomes. Unexpected findings were that MI intervention had no significant effects in decreasing prenatal drinking behaviors. Intervention theory-based specific and nonspecific factors drive effective nursing interventions.
Osterman et al., 2014[32]	RCT of pregnant women (*n* = 122) from three prenatal clinics in the Midwest, who drank any amount of alcohol in the previous year; 64% African American. Intervention or no-intervention comparison group, pretest/posttest design.	MI was ineffective in decreasing drinking.

Use of Technology

Author	Design	Main Findings/Comments
Evans et al., 2012[34]	RCT of pregnant women (*n* = 123) first presenting for care at the Fairfax County, Virginia, Health Department. All with UC; Text4baby vs. UC alone.	There was a 73% retention rate at 28 weeks' gestation. Attitudes toward alcohol consumption improved from baseline to follow-up (*OR* = 2.57, 95% *CI* [1.13–11.24], *p* = .03).
Evans et al., 2014[35]	RCT of military women (*n* = 943); pregnant < 14 weeks, first presenting for prenatal care. All with UC; Text4 baby + UC vs. UC alone.	49% completed a 4-week follow-up survey. Beliefs about avoiding alcohol and attending health care appointments improved, but there were no changes in self-reported behavior.
Farrell-Carnahan et al., 2013[36]	Uncontrolled prospective pilot study of non–treatment-seeking community women at risk of AEP (*n* = 46). One-session MI-based AEP intervention via telephone with 3- and 6-month follow-up.	Reduction in drinking and improved use of effective contraception. Telephone may not be as potent as longer, face-to-face contact.
Wilton et al., 2013[37]	Women who screened positive for risky drinking and not using effective contraception (*n* = 131). RCT of intervention given via telephone vs. in person, followed for 6 months.	Women who were Black and unemployed were more likely to be randomized to in-person contact. Intervention modality was a significant predictor of outcomes. Overall, both groups had small but significant reductions in alcohol use and larger increases in use of effective contraception. 73% completion rate.
van der Wulp et al., 2014[38]	Dutch midwifery practices (*n* = 60) randomized to one of three conditions: health counseling, CT, or UC/routine alcohol care. CT patients received UC + three CT feedback letters. Health counseling was manual-driven with three feedback sessions. Follow-up at 3 and 6 months.	The CT group stopped using alcohol more often than the UC group. Health counseling was not given consistently, and reductions in alcohol use did not differ between CT and UC.
Ondersma et al. 2015[39]	Pregnant women (*n* =48) who screened positive for alcohol risk at an urban prenatal care clinic. RCT of e-SBI + three tailored mailings vs. control session on infant nutrition. Primary outcome was 90-day prevalence of abstinence.	Nonsignificant findings for underpowered pilot. e-SBI + tailored mailings were well received. Medium-sized intervention effects on 90-day period prevalence estimate.
Montag et al., 2015[40]	Non–treatment-seeking sample of American Indian/Alaska Native women of childbearing age (*n* = 263), randomized to online SBIRT or treatment as usual after assessment at 1-, 3-, and 6-month follow-up. One-third of sample was at risk for AEP.	Both the SBIRT and treatment as usual groups showed reduced alcohol use after enrollment.

Table 2: Study Summaries (cont)

Use of Technology (Continued)

Author	Design	Main Findings/Comments
Ingersoll et al., 2018[41]	Women ages 18–44, fertile, with Internet/telephone access, risky drinking, and at risk for unintended pregnancy for past 3 months (*n* = 71). RCT of 6-core automated, interactive, and tailored Contraception and Alcohol Risk Reduction Internet Intervention to static patient education.	64 of 71 women completed at least one part of 6-month assessment. The rate of unprotected sex significantly reduced from pretreatment to posttreatment and at 6-month follow-up. Reductions in risky drinking were seen from pretreatment to posttreatment, but not at 6 months. Rate of AEP pretreatment (67%) reduced to 32% posttreatment and 30% at 6 months. Using a combined pregnancy outcome variable, neither an intent-to-treat analysis nor a group by treatment analysis were statistically significant.
Wernette et al., 2018[42]	RCT of pregnant women (*n* = 50) at a prenatal clinic in a large inner-city hospital. Computer-delivered, single session brief motivational intervention + booster session addressing substance use disorder and significant risk for sexually transmitted infection vs. control group.	Intervention was acceptable to participants. The intervention group had a 54% reduction in marijuana and alcohol use compared to the control group (16%) (p = .0015). There was a nonsignificant reduction in vaginal sex without a condom.
Jussila et al., 2020[43]	RCT of pregnant women referred to a hospital OB outpatient clinic due to recent or current substance use (*n* = 90), all treatment as usual. Intervention groups: three interactive US, pregnancy diary, and three prenatal infant consultations. 4D US thought to enhance parental mentalization and prenatal attachment.	89% retention rate, intervention group > control. 74% attended all three US sessions. Women in the intervention group attended fewer OB sessions than those in the control group (59% vs. 83%, p = .02). Fetal drug exposure and perinatal outcomes were similar in both groups.

Note: 4D, four-dimensional; AEP, alcohol-exposed pregnancy; *CI*, confidence interval; CM, case management; CT, computer tailoring; EARLY, MI + assessment feedback; e-SBI, electronic screening and brief intervention; MI, motivational interviewing; NFP, nurse-family partnership; OB, obstetrics; *OR*, odds ratio; RCT, randomized controlled trial; SBI, screening and brief intervention; SBIRT, screening, brief intervention, and referral to treatment; TEP, tobacco-exposed pregnancy; UC, usual care; US, ultrasound.

Discussion and Conclusions

This narrative review summarizes the many well thought-out clinical trials and randomized clinical trials conducted in the last decade assessing four types of interventions to reduce preconception and prenatal alcohol use, the sole cause of FASD.

Although they were well intentioned, clinically appealing, and may help some individuals, case management and home visits did not appear to have strong empirical support. These efforts were challenging to implement even by the investigators and may be difficult to reproduce by others. Some studies were exploratory in nature and had small sample sizes and no comparison groups, whereas larger efforts did not result in definitive advantages for this intensive approach.[20-23]

Interventions aimed at women prior to conception, which generally were based on the Project CHOICES approach,[24] had similar outcomes. In these studies, the reduction in AEP risk was largely realized by improved contraception in women of childbearing age who were sexually active and drank alcohol while not pregnant.[25,27-30] Although the Project CHOICES-based efforts used well-defined strategies and designs, it is unknown whether these women refrained from alcohol use when they became pregnant in the future.[44]

Two studies of motivational interviewing to reduce prenatal alcohol use published in the past decade did not demonstrate the efficacy of the intervention.[31,32] Both studies were small, including fewer than 200 pregnant women combined. Another limitation was low baseline levels of alcohol use in the study sample, which allowed for little room for improvement.

Finally, several studies evaluating the impact of technology were reviewed. Most had small sample sizes as they were exploratory in nature. However, their contributions include preliminary evaluations of techniques such as text messages, telephone contact, as well as computer-based screening and motivational interviewing that may inform future research and clinical efforts, particularly as the COVID-19 pandemic has impacted how treatment may be delivered in the future.[33-42] A recent meta-analysis of six of the 10 studies included in this review supports the potential of digital interventions.[45]

Future Directions

The findings described in this review indicate the need for research to address the limitations of the evidence to date. As there may be other potential obstacles to progress, prevention of AEP and the incidence of FASD will require a multipronged approach. First, FASD is one of the most complex developmental disabilities, and although prenatal alcohol exposure is the proximal teratogen, it rarely occurs in isolation.[46] The etiology of FASD also includes a range of lifestyle, sociodemographic, maternal, social, gestational, and genetic factors that need to be considered.[3] Future studies may include systematic collection of prespecified variables selected by expert panels to ensure consistency and comparability of studies.

Second, many of the past studies have had a narrow focus that did not take into consideration the complexity of modern childbearing. For example, punitive laws associated with prenatal substance use may discourage patient disclosure and hence limit opportunities for clinical intervention.[47,48] There is continued controversy in some nations over whether the law should intervene when a pregnant woman's actions may risk serious and preventable fetal injury.[49,50] The full impact of punitive laws on alcohol and substance use by pregnant women remains to be fully evaluated in future research; however, it is notable that the United States does not criminalize or punish women with other health conditions (e.g., diabetes, epilepsy, obesity) that can affect their pregnancy or their children.[47]

Third, although it is desirable to base clinical practices on sound research, the demand for evidence-based practice may have unintended consequences in clinical work or the ability to offer guidance to patients and practitioners alike as long as a definitive approach remains elusive.[5,6,51] For example, some have concluded that the absence of a safe drinking limit during pregnancy means that some alcohol use during pregnancy is acceptable.[52,53] Others have recognized that the lack of evidence is linked with a lack of dedicated funding and services, and consequently a lack of policy formulation and strategic direction in the United Kingdom[54] and Africa;[55] other types of gaps have been identified in Canada.[56,57] Thus, the impact of research is potentially far-reaching. As such, it may be time to understand the limits of conventional research designed to optimize efficacy, and to consider the potential contribution of more pragmatic approaches.[58]

Finally, two problematic secular trends have emerged in recent years: the increase in alcohol use among women during the COVID-19 pandemic[59,60] and a growing mistrust of science and professional advice that has historical roots.[61,62] For example, reliance on information disseminated by organizations funded by the alcohol industry, including mandatory pregnancy health warning labels on alcohol, may reduce the willingness of pregnant women to stop drinking alcohol.[63,64] Thus, too many people remain unconvinced that prenatal alcohol use is unsafe, or they do not understand the more nuanced rationale for abstinence. Future research may need to take into account the social contexts of problematic or risky behaviors so that interventions can be effectively implemented.

With 14% of pregnant women reporting alcohol use between 2018 and 2020, the Healthy People 2030 goal of 92% prenatal abstinence is a stretch. To reach that goal and optimize pregnancy outcomes, the next steps may require bold and creative thinking that goes beyond the well-studied preconception approaches, such as evaluation of those using video and other technologies that are consonant with patients and treatment professionals alike. Collection of consistent data sets to advance knowledge also may be helpful to reach the goals.[3] The costs associated with FASD are high and may be even higher than currently realized;[65-67] therefore, additional efforts to identify effective ways to reduce the risk of AEP are clearly warranted.

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Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes: A Narrative Review and Methodological Considerations

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PURPOSE: Although abstinence is recommended in pregnancy, many pregnancies are exposed to alcohol. Observational studies of the effects of low to moderate prenatal alcohol exposure (PAE) and neurodevelopmental outcomes have yielded inconsistent results, with some studies finding an increased risk of adverse neurobehavioral and cognitive outcomes, and other studies finding no changes or reduced risk of the same outcomes. The purpose of this narrative review is to summarize these inconsistencies and apply a methodological framework to discuss how different parameters contribute to the findings. The authors also provide recommendations on how to advance future research in this area.
SEARCH METHODS: The PubMed, Web of Science, and Embase databases were searched, along with reference lists of selected systematic reviews and meta-analyses. Search terms used were (infant or child or children or adolescent or offspring) AND (low or light or mild or moderate or low-to-moderate) AND (drinking or alcohol or drinks) AND (pregnancy or prenatal or fetal) AND (neurodevelopment or behavioral or psychological or cognitive or developmental) NOT (mice or rat or fish or animal) NOT (meta-analysis or review). Peer-reviewed original research studies were included if they analyzed associations between an exposure defined and characterized as low/light or moderate PAE with offspring neurodevelopmental outcomes. Animal studies, studies that did not provide clear cutoff points to classify PAE categories, studies lacking an abstinence control group, and studies that did not present a multivariable-adjusted measure of association were excluded.
SEARCH RESULTS: The searches identified 2,422 papers, with 36 papers meeting eligibility criteria. These studies were carried out across nine countries and included samples ranging from approximately 500 to 40,000 participants. Cognitive, academic, socioemotional, and behavioral outcomes were assessed from infancy through age 19.
DISCUSSION AND CONCLUSION: When the findings from the selected articles were summarized by geographic region, exposure definition, or neurodevelopmental outcome, no consistent observations or patterns emerged between low to moderate PAE and offspring outcomes. Although some studies found positive (i.e., beneficial) associations between low to moderate PAE and outcomes (primarily outcomes related to cognition) and others found negative (i.e., detrimental) associations (primarily for behavioral outcomes), most findings were null (i.e., showed no effect of PAE). The heterogeneity in study results is likely due to methodological issues, including residual confounding, effect measure modification, and exposure misclassification that make synthesis of studies difficult. Alternative study designs, including longitudinal trajectory analysis, sibling design, negative controls, and instrumental variable analyses, may reduce biases and are discussed. To date, the consequences of light to moderate levels of PAE on neurodevelopment remain unresolved; studies that advance methodological rigor will be important contributions to the field.

KEYWORDS: alcohol; prenatal alcohol exposure; fetal alcohol spectrum disorders; epidemiology; low, light, or moderate exposure; neurodevelopment

Prenatal alcohol exposure (PAE) is a necessary cause of fetal alcohol spectrum disorders (FASD), a group of alcohol-related conditions characterized by neurodevelopmental problems. Although PAE is associated with many adverse physical, neurodevelopmental, and social outcomes, the most commonly studied are neurodevelopmental—primarily behavioral and cognitive—outcomes. Associations between heavy PAE (which is inconsistently defined) or binge PAE (defined as consuming four or more drinks in about 2 hours in women, or the amount of alcohol necessary to achieve a blood alcohol concentration of 0.08% or higher[1]) and adverse neurodevelopmental outcomes have been well documented in the literature.[2-4] However, findings regarding associations between lower levels of PAE and neurodevelopmental outcomes are inconsistent, with summations to date yielding, at best, inconclusive results.[5] Moreover, there is no consensus in the literature on the definition of "low to moderate" PAE—or, correspondingly, the level of harm that low to moderate PAE may cause[5]—leaving pregnant individuals and their clinicians ill-equipped to assess risk of exposure.

Several systematic reviews[6-10] and at least four meta-analyses[2,11-13] have assessed associations between low to moderate PAE and child neurodevelopmental outcomes. Pooling results from studies published through 2012, Flak and colleagues reported a small positive association between mild to moderate PAE (defined as up to six drinks per week) and child cognition (beta estimate 0.04; 95% confidence interval *CI* [0.00, 0.08]); seven studies).[2] They also identified a modest association between moderate PAE (defined as up to six drinks per week, including some individuals who consumed at least three drinks per week) and adverse behavioral outcomes, such as problems with behavior regulation and increased demand for attention at ages 9 months to 5 years (beta estimate -0.15; 95% *CI* [-0.28, -0.03]; three studies).[2] A more recent meta-analysis, pooling studies published through 2020, also found that low to moderate PAE (author characterized, or one to fewer than seven drinks per week) was associated with adverse behavioral outcomes (i.e., attention problems) at ages 6 to 17 (*OR* 1.21; 95% *CI* [0.88, 1.65]; six studies). However, the magnitude of associations estimated varied dramatically across studies.[11] Dissimilar to the prior studies, a meta-analysis specifically examining the effect of low to moderate PAE (≤ 20 g/week to ≤ 50 g/week) on risk of attention-deficit/hyperactivity disorder (ADHD) reported no effect (*OR* 0.96; 95% *CI* [0.86, 1.02]; six studies).[12] Studies included in this review used a few different measures to assess ADHD symptoms between ages 3 and 14.[12]

The impetus remains to better understand the relationship between low to moderate PAE and offspring neurodevelopmental outcome. Although most authoritative bodies recommend complete abstinence from alcohol during pregnancy, PAE continues to be common, particularly in the early weeks of gestation prior to pregnancy recognition. In surveys conducted by the Behavioral Risk Factor Surveillance System between 2018 and 2020 in the United States, about 14% of pregnant women reported past 30-day alcohol use.[14] It is possible that the inconclusiveness in previous research findings is not driven by a paucity of research, but by inconsistencies in methodology used across studies. The purpose of this narrative review is thus threefold. First, it briefly summarizes select literature of low to moderate PAE and neurodevelopmental outcomes, noting consistencies and inconsistencies in findings. Second, it reviews methodological issues that limit valid ascertainment of the effects of low to moderate PAE on offspring neurodevelopmental outcomes. Third, it discusses alternative study designs that may address key methodological issues for consideration in future research.

Methods

Search Strategy

The PubMed, Embase, and Web of Science databases were searched on June 13, 2022. The search terms used to identify articles were (infant or child or children or adolescent or offspring) AND (low or light or mild or moderate or low-to-moderate) AND (drinking or alcohol or drinks) AND (pregnancy or prenatal or fetal) AND (neurodevelopment or behavioral or psychological or cognitive or developmental) NOT (mice or rat or fish or animal) NOT (meta-analysis or review). Results of the search strategy were checked against reference lists of existing systematic reviews and meta-analyses to verify that the search was comprehensive.[2,6,11,12]

Eligibility Criteria

The inclusion criteria for this review were: (1) peer-reviewed original research study; (2) human participants; (3) PAE characterized as low, light, mild, moderate, or low to moderate; and (4) any neurobehavioral or developmental outcome in offspring. Exclusion criteria were: (1) reference group other than "no PAE" or abstinence; (2) no parameters provided for the quantity or frequency of alcohol exposure that was used to classify individuals as having low, light, mild, moderate, or low to moderate PAE; (3) inability to separate PAE from co-occurrence with exposure to other substances; (4) no adjustment for confounding variables; (5) no measure of association presented for low/light or mild/moderate PAE categories (e.g., exposure was analyzed as a continuous variable); (6) quasi-experimental study design; and (7) alcohol exposure not specific to the pregnancy period.

Data Abstraction and Synthesis of Results

One of two reviewers examined titles and abstracts of each article to identify articles meeting criteria for full text review. During full text review, one of two reviewers abstracted the following information from each study: (1) author and year;

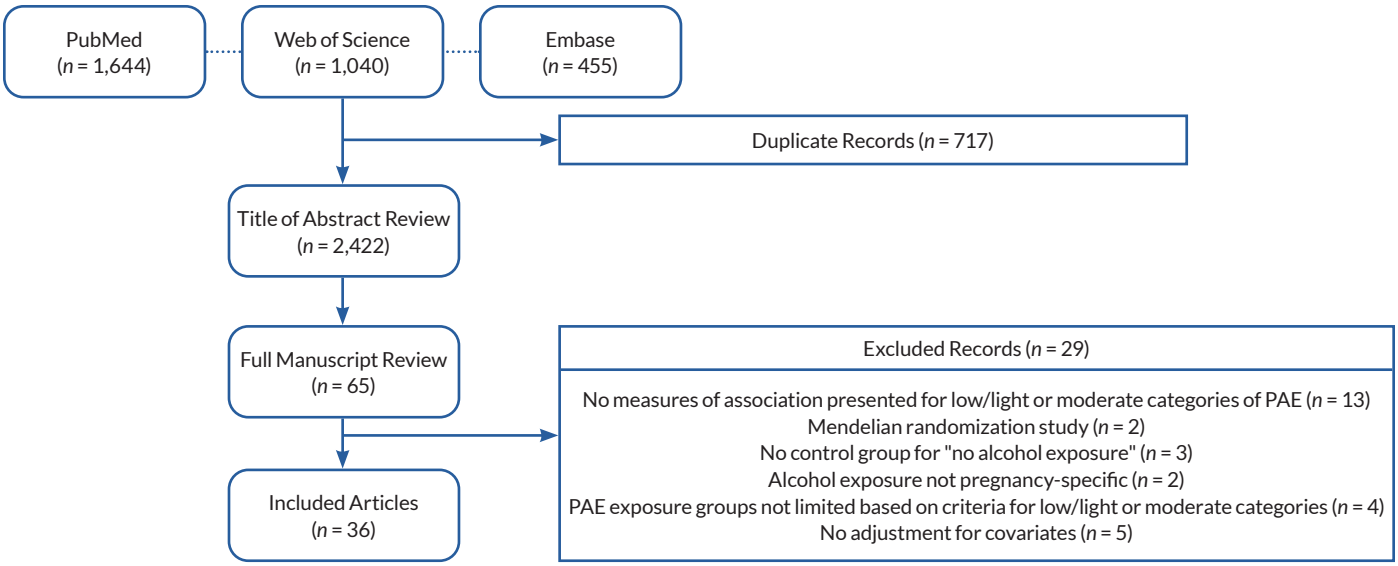

Figure 1. Sample selection for inclusion in this review. *Note:* PAE, prenatal alcohol exposure.

(2) data source and sample size; (3) study setting and offspring birth years; (4) definition of low/light PAE; (5) definition of mild/moderate PAE; (6) definition of one drink or one unit of alcohol; (7) timing of PAE measurement; (8) outcome, outcome measurement tool, and age of offspring at outcome measurement; (9) confounding variables considered; and (10) results. These data are summarized in Appendix 1.

Results

The search of PubMed (*n* = 1,644), Embase (*n* = 455), and Web of Science (*n* = 1,040) databases yielded 2,422 unique records, 65 of which met criteria for full text review. Of these, 29 were excluded (see Figure 1 for details of exclusions), and 36 were included in the final set. With a few exceptions, most studies reported no associations between low or moderate PAE and offspring neurodevelopment. Appendix 1 synthesizes the results by study location, exposure definition, timing of exposure measurement, and neurodevelopmental outcome.

Study Location

Included studies reported data from Australia (eight studies);[15-22] New Zealand, Australia, Ireland, and the United Kingdom (one study);[23] Denmark (nine studies);[24-32] Denmark and Finland (one study);[33] Japan (one study);[34] South Africa (one study);[35] the United Kingdom (nine studies);[36-43] and the United States (six studies).[44-49] Of note, many of the studies published from the same country used data from the same source. For example, half of the Australian studies used the Western Australian Health Survey data;[15,16,19,21] seven of the nine Danish studies used data from the Lifestyle During Pregnancy Study (based on a sample from the Danish National Birth Cohort);[26-32] and six of the nine U.K. studies used data from the Millennium Cohort Study.[36-39,41,50] Individual studies with the same data source often used the same definition of PAE but varied by study outcome or offspring age at neurodevelopmental assessment.

Some patterns were observed in study findings by country. For example, all studies from Denmark and Denmark/Finland reported mostly no effect of low to moderate PAE (i.e., null findings). Two studies—one using data from the Danish National Birth Cohort[25] and one using data from the Aarhus Birth Cohort[24]—published findings suggesting potential protective associations between low PAE and ADHD in both sexes[24] and between low to moderate PAE and internalizing problems among boys.[25] Results from each of the Australian studies were also mostly null, although two studies reported some evidence for worse behavioral problems with low PAE,[15,17] and three studies reported some evidence for better behavioral,[16] cognitive,[18] and academic outcomes with low PAE.[21] Studies from the United Kingdom focused on potential sex differences in the associations between low to moderate PAE and neurodevelopmental outcomes. Two studies using data from the Avon Longitudinal Study of Parents and Children reported worse behavioral rating scores among girls (but not boys) exposed to light PAE compared to children without PAE.[40] Two analyses using data from the Millennium Cohort Study reported that light PAE was associated with better cognitive scores[36,37] and lower behavioral scores[37] among boys relative to boys without PAE. Of the six studies from the United States, findings from three studies suggested worse behavioral outcomes with light to moderate PAE compared with no PAE,[45,46,48] one study noted a possible protective association

between light PAE and autism spectrum disorder (ASD),[44] and the remaining two studies showed null results[47,49] (see Appendix 1).

Prenatal Alcohol Exposure

There was tremendous heterogeneity in how low/light or moderate PAE were defined. Most studies included in this review defined these categories based on the average quantity of drinks consumed per week. Of these, low/light PAE was most frequently defined as averaging one to four units of alcohol per week, with specific definitions ranging from less than one unit per week to less than seven units per week. The studies using the higher threshold of fewer than seven units per week were mostly conducted in Australia, matching the definition of low-risk drinking for women.[15-17,20,21,52] Many studies also considered units per occasion in their drinking definition. For example, most studies using data from the Millennium Cohort study defined low PAE as "not more than one to two units per week or per occasion;"[36-38,50] and most studies using the Western Australian Health study defined low PAE as "not more than seven drinks per week and up to two drinks per occasion."[15,19,20,21,52] The study from South Africa considered only the number of drinks per occasion (mild to moderate: not more than three drinks in one sitting, never binge drinking),[35] and the study from Japan considered only frequency of drinking (low PAE: drinking rarely to one to four times per month).[34] For moderate PAE, most definitions were between three and 10 drinks per week. Some considered drinks per occasion in addition to drinks per week (range from more than two to five drinks per occasion).

The definition of a drink or unit of alcohol also varied by country. A standard drink was typically defined as 10 grams of pure alcohol in Australia; 12 grams in Denmark; and 8 grams or half a pint of beer, a glass of wine, or a single measure of spirits or liquor in the United Kingdom (see Appendix 1). No clear pattern of study findings emerged by definition of low/light PAE, moderate PAE, or unit of alcohol.

Timing of PAE Assessment

In all studies analyzed, PAE was assessed using self-report by the women as there are no biomarkers that could provide details on the dose and timing of low to moderate PAE. Twenty-two of the included studies collected self-report information on PAE during pregnancy,[16-18,20,22-33,35,40,42,43,47,49] and 14 studies relied on reports obtained after pregnancy,[15,19,21,34,36,37,39,41,44-46,48,50] including 10 studies with reporting occurring within 1 year of delivery.[15,19,21,36-39,41,45,50] Only three studies assessed PAE at the same time as or after the infant outcome was assessed (i.e., retrospectively). Of these, two reported that light to moderate PAE was associated with worse behavioral problems at age 23 months[48] and at ages 9 to 10 years;[46] and the third—a case control study examining associations with ASD—reported mostly null findings, with the exception of a protective association between one to two drinks in the first month of pregnancy and lower odds of ASD.[44]

Neurodevelopmental Outcomes

The studies included in this review assessed neurocognitive, academic, and behavioral outcomes when offspring were between the ages of 9 months and 19 years. None of the included studies reported worse neurocognitive outcomes with low to moderate PAE. For example, studies examining associations between PAE and Bayley Scales of Infant Development mental development and psychomotor indices in infants ages 12 to 24 months reported mostly null findings,[17,20,42,52] with one study suggesting that low PAE in the second and third trimesters was associated with better cognitive outcomes.[18] Three studies examining associations between PAE and IQ at about age 5 also reported null findings.[23,26,29] Four studies using data from the Millennium Cohort Study that assessed cognitive development with the British Abilities Scale reported protective associations between light PAE and cognitive outcomes in boys at age 3[36] and age 5,[37] but null associations when the boys were evaluated at ages 7 and 11.[38,39]

For measures of socioemotional and behavioral health, most studies used data ascertained with either the Strength and Difficulties Questionnaire (10 studies[25,30,33,36-41,43]) or the Child Behavior Checklist (CBCL; seven studies[15,16,22,23,34,35,46]). For these measures, offspring age at the time of assessment varied greatly and results were mixed with no clear pattern identified. Others studies examined PAE in relation to specific diagnoses, including ADHD (two studies)[24,41] and ASD (two studies),[44,50] with all studies reporting null or protective results. Three studies used infant behavior rating checklists to measure behavioral outcomes in infants ages 9 to 24 months. All three studies reported that light to moderate PAE was associated with worse behavioral outcomes in infancy, including increased infant difficultness,[48] poorer social engagement,[45] and more sensation seeking.[17]

Regarding academic outcomes, three studies reported null associations between PAE and academic achievement.[39,40,49] One study reported that low PAE was associated with lower odds of meeting Australian numeracy academic benchmarks, but had no effect on meeting reading, spelling, or writing benchmarks.[21]

Discussion and Comment

Despite a large and growing evidence base, making inferences about the effects of light to moderate PAE on neurodevelopmental outcomes in offspring remains difficult. Heterogeneity in effect estimates has persisted over the past 20 years, with both harmful and protective effects observed. Due to differences in study approach (summarized in Appendix 1) and methodological limitations, the diverse study results are difficult to synthesize. The following section further details these issues.

Methodological Issues With the Study of Light to Moderate PAE

Definition of exposure

Currently, there are no standard criteria or consensus for defining low/light or moderate levels of PAE. This heterogeneity in exposure definitions has undoubtedly contributed to inconsistencies of effect estimates and has limited the ability to make comparisons between studies. Even in abstracting data for this review, the authors were limited to papers that self-classified exposure into low/light or moderate PAE. Undoubtedly, many additional studies have investigated similar exposure levels but did not label them as such and therefore could not be included in this review because they were not identified in the literature searches.

Nondifferential exposure misclassification: Static measurement of PAE

Studies of PAE often use time-insensitive, or static, categorizations of exposure (e.g., categorizing the entire pregnancy as "high" consumption based only on PAE at conception) that fail to incorporate the dynamic changes in exposure that occur across pregnancy. These changes typically occur around the time of pregnancy recognition (which can be highly variable across individuals) when many women reduce their consumption or abstain from alcohol. For some pregnancies, changes also occur later in pregnancy as the perceived "risk period" for fetal development passes and women feel more comfortable resuming some level of alcohol use. The timing of these transition points may be informative with respect to offspring development but frequently is not examined. Further, when time-varying exposures are collapsed into static variables, a resulting exposure misclassification may lead to attenuation of effect estimates. This is particularly of concern when studying the consequences of low and moderate PAE, where modest effect estimates can disappear entirely due to exposure misclassification.

Differential exposure misclassification: Recall bias

There are no validated biomarkers that reflect prenatal exposure several years after birth; consequently, PAE is frequently measured by maternal recall. The gold standard approach in the collection of PAE information is through timeline follow-back[53] during pregnancy before the outcome is known, after which recall bias may occur. Due to stigma, individuals who perceive neurobehavioral problems in their offspring may be more likely to underreport their prenatal alcohol consumption relative to individuals who perceive no problems, which in cases of extreme underreporting could result in estimated protective effects of PAE. Although it is much more feasible to collect information retrospectively once children are at suitable ages for neurodevelopmental evaluation, the impact of this approach on internal validity must be critically evaluated.

However, when assessing studies that rely on recalled PAE, research has noted that recalled alcohol exposure is strongly predictive of pregnancy, dysmorphic, and neurodevelopmental outcomes.[54] Further, validation studies comparing prospective and retrospective reports found that retrospective reports of maternal drinking reflect higher levels of consumption than prospective reports obtained during the prenatal period.[55-58] Thus, although collection of consumption data during pregnancy remains preferable, retrospective information should not be discounted simply due to potential recall bias, and actually may be more accurate in some groups, particularly those who perceive stigma when reporting during pregnancy. Notably, however, the ability to recall PAE after pregnancy may differ by level of prenatal alcohol use. Women who did not consume any alcohol as well as those who habitually had high levels of consumption both may have more accurate recall than women who infrequently consumed alcohol, particularly with respect to the precise timing and amount of alcohol. This differential exposure misclassification could move estimates of PAE effects in either direction (toward or away from the null).[59]

Confounding variables

To validly estimate a causal effect of PAE on neurodevelopment, the exposure groups must be interchangeable. This means that variables that could confound the association between light to moderate PAE and offspring outcomes are equally balanced between alcohol-exposed and non–alcohol-exposed offspring. In observational studies, many socioeconomic and psychosocial factors have been associated with alcohol consumption patterns. A study of more than 6,000 women in Australia examined maternal factors associated with patterns of alcohol consumption before, during, and after pregnancy. The analysis found that compared to women with light prenatal alcohol consumption (0.4 drinks per day pre-pregnancy, early pregnancy cessation), women with high levels of alcohol consumption (2.5 drinks per day pre-pregnancy, 0.6 drinks per day during pregnancy) were more likely to have a lower income, be single or divorced, be pregnant for the first time, not attend church, report depression or anxiety, have high maternal adversity, and have adverse health-related lifestyle behaviors (e.g., smoking, little exercise, poor sleep).[60] Within the same study, women who abstained from alcohol pre-pregnancy through postpartum were also more likely to have lower income, have more children, and have adverse health-related lifestyle behaviors compared to women with light prenatal consumption.[60] Similarly, in a study of 4,000 pregnant women in the United Kingdom, women of higher socioeconomic status were more likely to drink wine, which was more likely to be consumed in low to moderate amounts, and less likely to binge drink than those with lower socioeconomic status. Further, being older, being better educated, having a higher social class, being employed, and having a better educated, employed partner were associated with consumption of wine, whereas smoking, lower education, and worse mental health

were more strongly associated with consumption of beer.[61] Other factors that favor positive neurobehavioral outcomes in the offspring were disproportionally shared by women who consumed low to moderate amounts of alcohol. These factors include better diets, earlier use of prenatal vitamins, lower prevalence of mental illnesses such as depression or anxiety, and lower likelihood to use other substances (e.g., marijuana) during pregnancy.[62] These studies highlight the imbalance in protective factors that often accompany low to moderate PAE that may bias findings, resulting in null or even protective effects when compared to abstinence.

Although these and other factors associated with low to moderate prenatal alcohol consumption that reduce the likelihood of adverse neurodevelopmental outcomes are routinely included in multivariable adjustment, they may still result in unmeasured confounding. Additionally, sample sizes typically limit the ability to include the multitude of variables necessary to establish true exchangeability. As a result (and common in all observational studies), the degree to which residual confounding persists—as evidenced by positive associations often reported between low to moderate PAE and offspring neurodevelopmental outcomes—must be considered because there is no conceivable benefit on these outcomes from PAE itself.[63,64]

Effect modification

In addition to confounding, which affects the internal validity (bias) of an estimate, the effect modification of the estimate by factors associated with the outcome (which can be termed modifiers or moderators) must be considered. Although not a source of bias, this occurs when the magnitude of the effect varies across levels of a third variable, which may contribute to heterogeneity in effect estimates across studies. Here, the authors hypothesize higher socioeconomic status associated with low to moderate PAE to be the third variable. For example, women consuming low to moderate levels of alcohol in pregnancy, which as previously noted is associated with higher socioeconomic status, may have more resources at their disposal postnatally compared to either women who abstain or women who consume large quantities of alcohol, providing a more enriched environment for the offspring. These factors include the likelihood to breastfeed, high-quality childcare, reduced environmental exposures, higher levels of social support, access to health care, reduced caregiver stress, and educational resources available to the child, which benefit neurodevelopmental outcomes. When effect estimates for PAE are not stratified by these potential modifiers, outcomes most vulnerable to PAE may be masked by the preponderance of protective factors associated with low to moderate PAE.

In summary, the null or protective effects attributed to low to moderate PAE on neurodevelopmental outcomes are likely, at least in part, attributable to unmeasured confounding associated with the exposure as well as to effect modification by postnatal factors that favor children with low to moderate PAE. Many statistical tools exist to address confounding (e.g., propensity score adjustment, inverse probability of treatment weights) and effect modification (e.g., stratification, re-weighting to a standardized sample). However, these options are imperfect because of the strong psychosocial patterning of PAE and insufficient resources to validly measure all potential confounding variables or examine associations across population subgroups. Further, as previously noted, there is a high degree of nuance in the operationalization of PAE, highly likely leading to misclassification of exposure and attenuation of results. To overcome these challenges, researchers have begun applying novel study designs and exposure models to this research. The following section highlights a few such approaches.

Alternate Study Designs to Address Methodological Issues

The study designs and methodologies discussed in this section—which are by no means exhaustive—specifically target some of the aforementioned threats to validity. Thus, exposure misclassification can be addressed by determining longitudinal trajectories of PAE, and confounding can be limited through use of instrumental variables, sibling designs, and negative control studies. These methods can be used alone or in combination to enhance estimation of causal effects.

Longitudinal trajectories of PAE

Efforts to better capture and operationalize the three parameters of alcohol use (timing, dose, and duration) have been ongoing for many years. Initially, researchers manually clustered individuals based upon these characteristics. In one study, investigators created a composite measure of PAE that incorporated dose, pattern, and timing of consumption into a descriptive, categorical variable (e.g., low, moderate, binge drinking less than once per week; binge drinking once or twice per week; high PAE).[15] When they compared outcomes using these classifications to traditional analytic methods (i.e., average quantity per trimester, average daily exposure across pregnancy, average weekly exposure across gestation), the researchers detected increased odds of anxiety or depression for children with moderate PAE in the composite models that were not evident in the traditional analyses.[15] Lately, unsupervised machine learning techniques have been incorporated into analyses to identify patterns of use across gestation.

Although several methodologies exist, the underlying premise of longitudinal exposure modeling is to create groups with similar longitudinal exposure patterns to minimize heterogeneity within the assigned trajectory group and maximize heterogeneity between trajectories. In a study employing PAE trajectories in Ukraine, sustained alcohol exposure, even at relatively low levels (about one drink per day), was associated with modest

reductions in neurodevelopmental performance at 6 and 12 months of age compared with trajectories of higher PAE with alcohol reduction or cessation earlier in pregnancy.[65] In the Safe Passage Study (South Africa and the United States), the sustaining trajectory (modeled as maximum drinks per drinking day) was associated with sudden infant death syndrome, whereas the trajectories with similar early pregnancy consumption but earlier cessation were not.[66] By modeling trajectories, researchers can disaggregate patterns of consumption, even among individuals with low to moderate levels of consumption, to better identify and understand the nuanced risk of adverse offspring outcomes that are often lost when exposure variables are operationalized as static measures.

Instrumental variables

Instrumental variable (IV) analysis is another study design that could improve research on low to moderate PAE and address issues of exchangeability. An IV affects the outcome only through its effect on the exposure and is unrelated to potential confounders of the exposure–outcome association.[67] In experimental studies, the treatment assignment is the IV. However, when experimental studies are not feasible, researchers may use a "quasi-experimental" approach to an IV through "Mendelian randomization," involving the use of genetic variants that influence the exposure but are unrelated to factors that confound the exposure-outcome relationship.[68] Three recent studies using data from the Avon Longitudinal Study of Parents and Children (ALSPAC) utilized this design.[61,62,68] In one study, investigators used analysis of a maternal genetic variant in the alcohol dehydrogenase gene *ADH1B* as an instrument for assessing PAE.[61] Individuals who carry the rare variant rs1229984 in *ADH1B* have greatly increased enzymatic activity in the oxidation of ethanol to acetaldehyde. This increased activity results in a faster reduction of blood alcohol levels and sharper rise of acetaldehyde in blood and organs, leading to symptoms such as increased heart rate and nausea. Individuals with this variant consume less alcohol, and correspondingly, the fetuses of mothers with the variant have lower PAE. These observations were born out in the ALSPAC data, making this a suitable IV candidate for low to moderate PAE.[61,62,68] When the researchers used a traditional analysis of the effects of any alcohol exposure, estimates were largely null, indicating no effect. In contrast, when they stratified the analysis by type of exposure (i.e., preferred type of beverage), they noted positive effects between wine consumption and offspring academic achievement, and negative effects between beer consumption and offspring academic achievement. Given that there should be no difference between the effects of beer and wine when both were converted into standard, equivalent doses, the researchers suggested that the different outcomes were due to the strong social gradient associated with choice of beverage. *ADH1B*, however, was unrelated to potential confounders, but was predictive of alcohol use in pregnancy and, by extension, alcohol exposure to the developing fetus. When authors conducted the IV analysis with *ADH1B*, they found negative effects between PAE and academic achievement at all ages analyzed (i.e., ages 7 to 16).[61] Similar results were obtained when repeating this analysis with cognitive and educational performance at age 8.[68]

The third study that utilized an IV analysis constructed the IV from the child's genotype (variants of the alcohol dehydrogenase enzyme). It was hypothesized that offspring alleles, which result in "fast" metabolism of ethanol, would protect against abnormal brain development in infants.[62] Among offspring born to women with moderate alcohol consumption (one to six units per week during pregnancy), negative associations existed between the presence of genetic variants associated with slow alcohol metabolism and IQ at age 8. These associations were not seen in children with no PAE.[62] Assuming these findings can be replicated, they are a promising avenue to control for confounding factors while estimating the effects of low to moderate PAE on child health. Researchers should continue to explore additional IVs, specifically searching for stronger IVs than the alcohol metabolism variants, which can be exploited in this framework.

Sibling controls

A third promising approach that also addresses issues of exchangeability is a sibling design. In their simplest form, sibling designs compare outcomes among exposure-discordant siblings, which accounts for many shared environmental and familial confounders that are exceedingly difficult to fully adjust for through multivariable analysis. Sibling studies often result in attenuated effect estimates between prenatal exposures and offspring neurodevelopmental outcomes compared to traditional study designs,[69] highlighting the challenges of residual confounding.

At least three studies have utilized sibling designs to control for shared genetic and environmental confounders when studying PAE.[34,70,71] In the first sibling study conducted on approximately 4,000 mother-sibling triads, researchers found that employing the sibling design attenuated the initial multivariable-adjusted results for PAE and attention/impulsivity problems in offspring. However, an association still existed between heavy PAE (≥ 5 days/week) and offspring conduct problems at ages 4 to 11.[70] In a second study, conducted with 15,000 mother-sibling triads in Norway, researchers detected no effects of low levels of PAE on offspring behavioral problems, and attenuated yet modest effects of "hazardous" PAE on behavioral problems at age 3 when accounting for siblings.[71] A third study, which included 1,600 sibling pairs in Japan, found that low PAE (drinking one to four times per month or rarely) was associated with greater anxiety problems and internalizing problems. In that study, effect estimates were magnified in the siblings analysis compared to initial multivariable-adjusted

results.[34] Although researchers must consider exposure discordance and factors that changed between births (e.g., birth order, maternal age, socioeconomic factors) that may bias results, this model is a promising strategy to mitigate the persistent problem of residual confounding.

Negative controls

Finally, another option that does not require identifying an IV or observing siblings is to employ a negative control design. Such designs compare the effects of PAE on offspring outcome with the effects of similar exposure with no biological relevance to the offspring (e.g., maternal exposure prior to conception or postnatally or similar levels of exposure of other individuals [partner exposure]).[67] Negative control designs alert the analyst to uncontrolled confounding because if any of the effect estimates among the negative controls are positive, the effect measure of interest is likely confounded. Putting this design to practice, researchers using paternal exposure during pregnancy as a negative control in the Avon Longitudinal Study of Parents and Children found no evidence that maternal alcohol and tobacco consumption during pregnancy were more strongly associated with childhood IQ than paternal alcohol and tobacco consumption.[72] In a second negative control study using ALSPAC data, researchers found that offspring of mothers who consumed any alcohol at 18 weeks of gestation had a 17% increased risk of having a diagnosis of depression at age 18.[73] There was no clear evidence of association between partners' alcohol consumption at 18 weeks of gestation and increased risk of offspring depression. The investigators concluded that the negative control comparison of paternal drinking provided some evidence that the association between PAE and depression at age 18 may be causal and warranted further investigation and replication.[73] Although one must critically evaluate the potential for causality by the selected negative control (e.g., epigenetic effects in the case of paternal exposure), if it is deemed that there is no plausible mechanism by which the negative control could affect the outcome, the types of analyses should be conducted and reported.

Conclusions

Limitations of This Review

When reviewing the findings presented here, several limitations should be considered. First, restricting findings to published research introduces the potential for publication bias. However, given that many studies reviewed here yielded null findings, it is unlikely that publication bias greatly affects the results. Second, the high degree of heterogeneity in methods and the exposure definition prevented a meta-analysis.

Third, and most importantly, although three databases were searched, this review is a narrative review only and should not be interpreted as a systematic review. The search process used search terms "low, light, mild, or moderate," and the authors then looked for predefined criteria for the categorization. Accordingly, there could be additional studies that included similar exposure ranges but did not use those terms; other studies may have included a population with a mean PAE that fell within a range described as low or moderate, but the study did not actually set minimum or maximum parameters for defining the exposure. Either scenario would have resulted in exclusion of those studies from this review. For example, Parry et al. examined several categories of alcohol exposure (i.e., 0 g/week, > 0–29 g/week, 30–59 g/week, 60–89 g/week, 90–119 g/week, and > 120 g/week)[74] that overlap with definitions of low/light or moderate PAE in many of the studies included here. However, the study was excluded because the authors did not label these categories as low, moderate, or high exposure. Similarly, Beauchamp et al. enrolled a birth cohort with a PAE group that had a median of 0.84 ounces absolute alcohol per day during the periconceptual period and 0.3 ounces absolute alcohol per day during pregnancy.[75] Although this sample may have overlapped with other samples in this review of moderate PAE, it likely also included infants who would have been categorized as low PAE and some who would have been categorized as high PAE, given that no minimum or maximum criteria were set for inclusion. Therefore, the study was excluded from this review. Attempting to include all such studies would require (1) searching for any study that had any measure of PAE, and (2) having clearly defined and accepted standards for categorizing low and moderate PAE. Such manual abstraction and classification in the absence of consensus definitions were beyond the feasibility of this review.

Fourth, the studies reviewed here assessed a variety of neurodevelopmental outcomes that may differ in sensitivity to alcohol based on the measure or the timing of administration. It was beyond the scope of this review to comment on each measure's sensitivity and psychometric properties, although both also may contribute to the heterogeneity in findings. Finally, this review was limited to neurodevelopmental outcomes. There are other health and behavioral outcomes potentially affected by PAE that manifest across the life course and also warrant systematic investigation.

Public Health and Clinical Implications

Although pregnant women may want to know whether there is a safe drinking threshold in pregnancy, this question is difficult to answer in human research. As seen in this review, findings are heterogeneous across studies, and many methodological limitations impair ability to validly estimate the potential consequences of low to moderate PAE. Moreover, the public should not confuse inconsistent evidence and insignificant findings to indicate absence of an effect. Accordingly, the only way to be certain to avoid adverse outcomes associated with alcohol exposure is to follow current guidelines to abstain from alcohol during pregnancy. Given that many women do not

plan pregnancy, those who have had alcohol exposure prior to learning they are pregnant should avoid continued use.

Summary and Future Directions

Although this review generally found null associations between low to moderate PAE and adverse neurodevelopment, the issue is far from resolved. There is no consensus in the literature on the level of harm that low to moderate prenatal alcohol exposure may cause, and the differential vulnerability resulting from influences such as concurrent exposures to other substances, genetics, and other factors may prevent a clear conclusion. Although the evidence base continues to expand, substantial methodological limitations impair synthesis of study findings. Use of alternative study designs may help advance research of the effects of low to moderate PAE on adverse outcomes, and the authors look forward to the expansion of these methodologies in the field. In addition, expanding reviews to capture other outcomes, including physical and behavioral outcomes, as they emerge across the life course is of great interest.

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Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Barbuscia et al. (2019)[39]	Millennium Cohort Study (*n* = 10,454)	United Kingdom 2000-2002	< 1-2 units per week per occasion	< 3-6 units per week or < 3-5 units per occasion	Half-pint of beer, glass of wine, or single measure of spirits or liquor	Interview at 9 months postpartum	British Ability Scale (BAS); ages 3, 7, and 11 Bracken School Readiness Assessment; age 5 Strengths and Difficulties Questionnaire (SDQ); ages 3, 5, 7, 11, and 14	Child gender, mother's ethnic background, low birth weight, maternal health, sociodemographic characteristics, and maternal cognitive ability	Light PAE was not associated with cognitive ability, internalizing, or externalizing behavioral problems. Moderate PAE was associated with better cognitive ability on 1 of 9 measures (others null) and worse externalizing problems at age 11 (null at other ages).
Bay et al. (2012)[27]	Danish National Birth Cohort Lifestyle During Pregnancy Study (*n* = 685)	Denmark 1997-2003	Low to moderate: weekly alcohol intake 1 to 14 drinks per week Analyzed as categories: 1 to 4 drinks per week 5 to 8 drinks per week 9 to 14 drinks per week		12 g pure alcohol	Self-reported at 17 weeks of gestation	Movement Assessment Battery for Children; age 5	Paternal education, maternal IQ, prenatal maternal smoking, maternal age, parity, maternal binge drinking episodes during pregnancy, prenatal and postnatal marital status, postnatal parental smoking, maternal pre-pregnancy body mass index (BMI), child sex, age at testing, health status, hearing and vision on day of testing, family/home environment, physical activity	No association existed between low to moderate PAE and total motor impairment or any motor skill subscales.
Chen (2012)[48]	National Longitudinal Survey of Youth (*n* = 1,618)	United States 1986-2000	Light to moderate: < 3 or 4 days a month		Not reported	Maternal report postpartum; < 23 months	Modified Rothbart Infant Behavior Questionnaire; ages < 23 months	Sibling fixed effects model with control for prenatal smoking, poverty status, marital status during pregnancy, prenatal care in first trimester (T1), parity, child sex	Light to moderate PAE was associated with greater difficultness of infant. No associations were found with positive mood of fearfulness subscales.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Cluver et al. (2019)[35]	Safe Passage Study (*n* = 500)	South Africa 2007-2015	Mild to moderate: ≤ 3 drinks in one sitting; never binge		14 g ethanol	Modified alcohol timeline follow-back around conception, up to 4 times during pregnancy, and at 1 month postpartum	Kaufman Assessment Battery for Children (KABC-II), a developmental neuropsychological assessment (NEPSY-II), Preschool Child Behavior Checklist (CBCL); age 4	Maternal age, education, smoking, marijuana use, and methamphetamine use	No associations existed with neurodevelopmental or behavioral outcomes.
Falgreen Eriksen et al. (2012)[29]	Danish National Birth Cohort Lifestyle During Pregnancy Study (*n* = 1,628)	Denmark 1997-2003	1 to 4 drinks per week	5 to 8 drinks per week	12 g pure alcohol	Self-reported at 17 weeks of gestation	Wechsler Preschool and Primary Scale of Intelligence—Revised; age 5	Parental education, maternal IQ, maternal smoking in pregnancy, child's age at testing, child's gender, parity, maternal marital status, maternal age and BMI, maternal binge drinking in pregnancy, family/home environment, prenatal/postnatal smoking, child's health status, hearing and vision abilities	No association existed between low or moderate PAE and IQ.
Forrest et al. (1991)[42]	Dundee antenatal clinics (*n* = 592)	Scotland 1985-1986	Mild: 1-49 g per week	Moderate: 50-99 g per week	10 g absolute alcohol (AA) (equivalent to one glass wine, half-pint beer, or one standard measure spirits)	Interviewer-administered questionnaire; T1, second trimester (T2)	Bayley Scales of Infant Development (BSID) mental development index (MDI) and psychomotor development index (PDI); 18 months	Maternal cigarette consumption, age, and social class; child's sex, birth weight, and gestational age	Mild PAE in early pregnancy was not associated with MDI or PDI. Moderate PAE in early pregnancy was not associated with MDI or PDI.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Gallagher et al. (2018)[50]	Millennium Cohort Study (*n* = 12,595)	United Kingdom 2000-2002	≤ 1-2 units per week or at any one time in pregnancy	≤ 3-6 units per week or 3-5 units at any one time in pregnancy	Half-pint beer, glass wine, or single measure spirits or liquor	Interview at 9 months postpartum	Maternal report of autism spectrum disorder (ASD) diagnosis; age 11	Parental age, household income, maternal education, social deprivation, ethnicity, marital status, maternal smoking, BMI, hypertension, diabetes, depression treatment, maternal smoking	No association existed between light or moderate PAE and ASD.
Goldschmidt et al. (2004)[49]	Maternal Health Practices and Child Development Study (*n* = 608)	United States 1984-1987	Light/moderate: < 1 drink per day		Not reported	Maternal interview at gestation months 4 and 7, and postnatally	Wide Range Achievement Test–Revised and Peabody Individual Achievement Test–Revised; age 10	Sociodemographic status, child characteristics and environment, maternal psychosocial status, current maternal substance use, and prenatal tobacco and illicit drugs other than marijuana	No association existed between light to moderate PAE and academic performance.
Halliday et al. (2017)[17]	Asking Questions About Alcohol in Pregnancy (*n* = 554)	Australia 2011-2012	≤ 20 g AA per occasion and ≤ 70 g AA per week	21-49 g AA per occasion and ≤ 70 g AA per week	10 g alcohol	Self-reported questionnaire at 13 weeks, 26 weeks, and postnatal interview; analyzed as T1, T2, and third trimester (T3)	BSID, Brief Infant-Toddler Social and Emotional Assessment; Infant/ Toddler Sensory Profile; age 2	Maternal age, maternal education, household income, ethnicity, language spoken in home, pre-pregnancy BMI, folic acid supplementation, smoking in pregnancy, age started drinking regularly, paternal drinking	Low to moderate PAE was not associated with most neurodevelopmental outcomes. Low PAE in T1 with abstinence in T2 and T3 was associated with more sensation seeking.
Hutchinson et al. (2019)[20,52]	Triple B Pregnancy Cohort Study (*n* = 1,324)	Australia 2009-2013	≤ 7 drinks per week and ≤ 2 drinks per occasion	≤ 7 drinks per week and > 2 to ≤ 4 drinks per occasion	10 g alcohol	Interview during T1, T2, and at 8 weeks postpartum; analyzed as T1, T2, and T3	BSID-III gross motor development, age 12 months	Age at birth, education, Socio-Economic Indexes for Areas, state of residence, country of birth, single-parent household, Aboriginal and Torres Strait Islander status, native language, substance use, pregnancy anxiety, IQ, parity, BMI, gestational age	No association existed between low or moderate PAE and gross motor development.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Ichikawa et al. (2018)[34]	Japanese Study of Stratification, Health, Income and Neighborhood (*n* = 1,600)	Japan 2010-2013	Drinking rarely to 1 to 4 times per month	Not applicable (N/A)	Not reported	Self-reported at offspring age 2-18 (same age as outcome measurement)	CBCL; mean age 9 (*SD* 4.4, age range 2-18)	Sibling analysis and adjustment for child's age, sex, parent's age, education, working status, family income, prenatal smoking, domestic violence, parental drinking	Low PAE was associated with greater anxiety problems, internalizing problems, and overall problems. Low PAE was not associated with several other CBCL-measured outcomes (e.g., externalizing behavior).
Kelly et al. (2009)[36]	Millennium Cohort Study (*n* = 9,460)	United Kingdom 2000-2002	≤ 1-2 units per week or per occasion	≤ 3-6 units per week or 3-5 units per occasion	Half a pint of beer, a glass of wine, or a single measure of spirits or liquor	Interview at 9 months postpartum	SDQ, BAS; age 3	Child's age, birth weight, mother's age at the time of birth, number of children in the household, mother's education, mother's drinking, mother's smoking habits, household income, pregnancy planned, mother's occupational class, mother's Kessler Psychological Distress Scale (K6) score, warmth of relationship between mother and child, parental discipline	Light PAE was not associated with behavioral or cognitive problems. In boys, light drinking was associated with lower conduct and hyperactivity problems, and higher cognitive ability. Moderate drinking was not associated with any outcome in either girls or boys.
Kelly et al. (2012)[37]	Millennium Cohort Study (*n* = 11,513)	United Kingdom 2000-2002	≤ 1-2 units per week or per occasion	≤ 3-6 units per week or 3-5 units per occasion	Half-pint of beer, glass of wine, or single measure of spirits or liquor	Interview at 9 months postpartum	SDQ, BAS; age 5	Child's age, birth weight, mother's age at time of birth, number of children in the household, mother smoked during pregnancy, pregnancy planned, parental income, highest parental educational qualification, highest parental occupation, mother's K6 score, parental discipline, child made to follow instructions, mother's parental competence, closeness of relationship between mother and child, mother's current drinking	No associations existed between light or moderate PAE and behavioral problems in girls or boys. Light PAE was associated with better cognitive ability scores in boys, null in girls; moderate PAE was not associated with cognitive ability scores.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Kelly et al. (2013)[38]	Millennium Cohort Study (*n* = 10,285)	United Kingdom 2000-2002	1-2 units per week or per occasion during pregnancy	N/A	Half-pint beer, glass of wine, or single measure of spirits or liquor	Interview at 9 months postpartum	SDQ, BAS; age 7	Mother's age, planned pregnancy, maternal smoking, parity, ethnicity, lone-parent family, life satisfaction, relationship quality, social networks, number of children in household, child's age, highest parental educational qualification, parental income, mother's mental health, parental discipline strategies, mother's self-rated competence, mother's closeness with child, mother's current drinking	No association existed between light PAE in pregnancy and behavioral or cognitive development.
Kesmodel et al. (2012)[26]	Danish National Birth Cohort Lifestyle During Pregnancy Study (*n* = 1,628)	Denmark 1997-2003	1 to 4 drinks per week	5 to 8 drinks per week	12 g pure alcohol	Interview at 17 weeks	Behavior Rating Inventory of Executive Function, Wechsler Preschool and Primary Scale of Intelligence—Revised, Test of Everyday Attention for Children at Five; age 5	Parental education, maternal IQ, prenatal maternal smoking, child's gender, child's age at testing, test administrator, parity, maternal marital status, maternal age, maternal BMI, prenatal maternal average number of drinks per week, home environment, postnatal parental smoking, health status, hearing and vision abilities	No association existed between low or moderate PAE and executive function, intelligence, or attention.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Kilburn et al. (2015)[32]	Danish National Birth Cohort Lifestyle During Pregnancy Study (*n* = 1,333)	Denmark 1997-2003	1-4 drinks per week	5-8 drinks per week	12 g	Interview at 17 weeks	Sternberg paradigm to assess information processing time and choice reaction time; age 5	Parity, prenatal maternal smoking, maternal pre-pregnancy BMI, length of parental education, marital status, postnatal parental smoking, child health status, family/ home environment index, breakfast irregularity, maternal depression, parental alcohol use, hearing ability, and vision ability	No association existed between maternal drinking and choice reaction time or information processing time.
Larkby et al. (2011)[47]	Clinical sample at maternity clinic (*n* = 592)	United States 1989-1991	Average drinks per day in T1 ≤ 0.4	Average drinks per day T1 > 0.4 to < 0.89	Not reported	Maternal report at 4th and 7th gestational months and at delivery	Computerized Diagnostic Interview Schedule-IV to measure conduct disorder; age 16	Prenatal exposure to tobacco, marijuana, cocaine, and other illicit drugs; income; race; gender; parenting style; life events; home environment; family history of alcohol problems; and maternal lifetime psychopathology	No association existed between light or moderate PAE and conduct disorder in adolescents.
Lees et al. (2020)[46]	Adolescent Brain Cognitive Development Study (*n* = 9,719)	United States 2005-2008	2.3 drinks per week (1st 7 weeks of pregnancy) 1.1 drinks per week through gestation	N/A	Not reported	Maternal report at offspring age 9-10 (same time as outcome measurement)	(1) CBCL, (2) Schedule for Affective Disorders and Schizophrenia for School-Age Children (K-SADS), (3) Impulsive Behavior Scale for Children–Short Form, (4) Behavioral Avoidance and Behavioral Inhibition Scales, (5) Cash Choice Task, (6) Rey Auditory Verbal Learning Test, (7) NIH Toolbox fluid intelligence battery, (8) brain imaging; ages 9-10	Birth weight, preterm birth, sex at birth, race/ ethnicity, youth age at time of assessment and school grade performance, maternal age at birth, maternal depression, and other substance use during pregnancy (tobacco, cannabis, cocaine)	Light stable PAE and light PAE reduced in pregnancy were associated with greater behavioral and psychological problems (CBCL: internalizing, externalizing, attention problems, total; K-SADS: anxiety, specific phobias) and differences in cerebral and regional brain volume.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Maher et al. (2022)[23]	SCOPE (Screening of Pregnancy Endpoints) and BASELINE (Babies After SCOPE: Evaluating Impact on Neurological and Nutritional Endpoints) (*n* = 1,507)	New Zealand, Australia, Ireland, United Kingdom 2004-2011	1-7 units per week		8 g or 10 ml (1 dl) pure alcohol	Interview at 15 weeks of gestation	CBCL; ages 2 and 5 Kaufman Brief Intelligence Test; age 5	Maternal age, maternal education, marital status, family income, maternal BMI, maternal smoking status at 15 weeks of gestation, and infant sex	No associations existed between low PAE and behavioral outcomes at age 2 or age 5.
McCormack et al. (2018)[18]	The Triple B Study (*n* = 1,331)	Australia 2008-2013	≤ 7 drinks per week and ≤ 2 drinks per occasion	≤ 7 drinks per week and > 2 to ≤ 4 drinks per occasion	10 g alcohol	Interview at T1, T2, and 9 weeks postpartum	BSID; age 12 months	Household SES, maternal age, maternal education level, Aboriginal or Torres Strait Islander origin, country of birth, single-parent household, first language, tobacco use, illicit substance use, anxiety, IQ, parity, and BMI	Low levels of PAE in T2 and T3 were associated with slightly higher cognitive scores. No associations existed with low or moderate PAE in T1. Moderate PAE in T2 and T3 was not analyzed.
Mitchell et al. (2020)[41]	Millennium Cohort Study (*n* = 13,004)	United Kingdom 2000-2002	≤ 3 to 7 drinks per week	≤ 8 to 14 drinks per week	Half a pint of beer, a glass of wine, or a single measure of spirits or liquor	Interview at 9 months postpartum	Parental report of attention-deficit/ hyperactivity disorder (ADHD) diagnosis, SDQ; age 7	Gender, gestational age at delivery, parity, paternal age, maternal age, maternal smoking status, maternal pre-pregnancy BMI, household income, maternal education, ethnicity, and marital status	Neither light nor moderate drinking were associated with ADHD, abnormal SDQ scores, or hyperactivity scores.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Niclasen et al. (2014)[25]	Danish National Birth Cohort (*n* = 37,152)	Denmark 1996-2002	Cumulative > 0 to 5 drinks per pregnancy; average > 0 to 2 drinks per week	Cumulative > 5 to 90 drinks per pregnancy; average 2 to 4 drinks per week	12 g pure alcohol	Maternal report at gestational week 16, week 30, and at 6 months postpartum	SDQ; age 7	Paternal smoking, parental education, parental pre-pregnancy psychiatric diagnoses, and maternal psychological well-being in pregnancy	No associations existed between light or moderate PAE with parent-rated conduct, emotional, hyperactivity/ inattention, or peer problems. Small protective associations existed between low and moderate PAE in early part of pregnancy with internalizing problems among boys (not girls).
O'Callaghan et al. (2007)[22]	Mater-University of Queensland Study of Pregnancy (*n* = 5,139)	Australia 1981-1984	Low: < 0.5 glass per day 0.5 to 1 glass per day		0.5 oz AA	Reported at first prenatal clinic visit and after delivery	CBCL; age 14	Cigarette smoking in early and late pregnancy, maternal BMI < 18.5, social risk score (low maternal education, maternal age < 19, single-parent status, or low income in pregnancy or at age 14)	No associations existed between light or moderate alcohol, neither early nor late in gestation, and attention, learning, or cognitive outcomes.
O'Leary et al. (2009)[19]	Western Australian Survey of Health (*n* = 1,739)	Australia 1995-1996	≤ 20 g alcohol per occasion, with a frequency of less than weekly up to 6 days per week	10 g to < 50 g alcohol per occasion, with a frequency ranging from less than weekly up to daily	10 g alcohol	Questionnaire at 12 weeks postpartum	Ages & Stages Questionnaire Communication Scale; age 2	McMaster's family functioning, parenting scale, partner present, maternal depression, anxiety, stress, maternal age at delivery, income, marital status, parity, education, smoking, use of tranquilizers, illicit drug use	No association existed between low PAE and language delay.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
O'Leary et al. (2010)[15]	Western Australian Survey of Health (*n* = 2,224)	Australia 1995-1996	< 70 g alcohol per week and ≤ 10-20 g per occasion	≤ 70 g alcohol per week and between 21 g and 49 g per occasion	10 g alcohol	Questionnaire at 12 weeks postpartum	Ages & Stages Questionnaire (language delay); age 2 CBCL; ages 2, 5, and 8	Antenatal covariates (maternal age, marital status, parity, ethnicity, income, maternal smoking, and use of illicit drugs, tranquilizers, and sleeping tablets during pregnancy), postnatal covariates (marital status, income, treatment for postnatal depression, postnatal depression, family functioning, parenting style, tension in the family due to alcohol and maternal depression, anxiety, and stress	No association existed between low PAE and language delay (also reported in paper above). Moderate PAE in T1 was associated with increased odds of anxiety/depression but not with somatic or aggressive problems. No association existed between low PAE and any CBCL outcome in children ages 2, 5, or 8.
O'Leary et al. (2013)[21]	Western Australian Survey of Health (*n* = 4,056)	Australia 1995-1996	1-2 drinks per occasion and < 7 drinks per week	3-4 standard drinks per occasion and ≤ 7 drinks per week	10 g alcohol	Questionnaire at 12 weeks postpartum	Western Australian Literacy Numeracy Assessment measures whether children met benchmarks for reading, writing, spelling, and numeracy; ages 8-9	Maternal age, education, marital status, ethnicity, parity, illicit and/or tranquilizer drug use, smoking, income, and languages spoken at home	Low PAE was associated with lower odds of missing numeracy academic benchmark; not statistically significant for reading, spelling, or writing benchmark. Moderate PAE was not associated with academic underachievement.
Robinson et al. (2010)[16]	Western Australian Pregnancy Cohort (*n* = 1,744)	Australia 1989-1991	Occasional: ≤ 1 drink per week Light: 2 to 6 drinks per week	7 to 10 drinks per week	10 g alcohol	Questionnaire at 18 and 34 weeks of gestation	CBCL; age 14	Maternal age, maternal education, presence of biological father in family home, family income, stress in pregnancy, maternal cigarette smoking, child's age	Light and moderate PAE in first 3 months of pregnancy was associated with lower internalizing and externalizing problem scores; no associations were observed for late pregnancy PAE.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Rodriguez et al. (2009)[33]	Three cohorts from Nordic Network on ADHD (*n* = 21,678)	Denmark, Finland 1984-2002	1 to 4 drinks per week	N/A	Not reported	Maternal report of average weekly exposure at ~16-32 weeks of gestation	SDQ, Rutter Scale; ages 7-15	Smoking, social adversity, birth weight, gestational age (analyzed in three cohorts to analyze differences in participant characteristics)	Low PAE was not associated with inattention/ hyperactivity.
Sayal et al. (2007)[43]	Avon Longitudinal Study of Parents and Children (*n* = 9,086)	United Kingdom 1991-1992	< 1 glass per week	N/A	8 g alcohol (equivalent to 1 glass)	Self-reported questionnaire at 18 weeks of gestation	Parental SDQ; 47 and 81 months Teacher SDQ; 92 to 108 months	Smoking, cannabis use, and use of illicit drugs in T1, highest level of maternal education, home ownership, marital status, parity, maternal age group, high Edinburgh Postnatal Depression Scale score, child ethnicity, gestational age group, and birth weight	Low PAE was associated with worse parental and teacher-rated SDQ scores among girls, but not among boys.
Sayal et al. (2013)[40]	Avon Longitudinal Study of Parents and Children (*n* = 10,558)	United Kingdom 1991-1992	< 1 glass per week	N/A	8 g alcohol (equivalent to 1 glass)	Self-reported questionnaire at 18 weeks of gestation	SDQ, Kay Stage 2 school examinations; age 11	Maternal age, parity, highest level of maternal education, daily frequency of smoking, use of cannabis and/or other illicit drugs during T1, home ownership, whether currently married, maternal mental health, child's gestational age, birth weight, and gender	No association existed between light PAE and outcomes. In girls, a suggestion of slightly worse outcomes appeared on parent-rated total SDQ score in those exposed to light PAE. Light PAE was not associated with Kay Stage 2 scores.
Singer et al. (2017)[44]	Study to Explore Early Development Case-Control Study (*n* = 2,515)	United States 2003-2006	Light: < 1 drink per week; 1-2 drinks per week		1 beer, 1 glass wine, 1 mixed drink, or 1 shot liquor	Maternal report* at 55 months postpartum (range: 29-68 months) *same time as outcome measurement	ASD diagnosis measured with the Autism Diagnostic Observation Schedule (child report) and the Autism Diagnostic Interview Revised (caregiver report); ages 30-68 months	Child's sex, total household income in the year prior to the pregnancy, self-reported maternal race/ethnicity, maternal education at delivery, maternal parity, at least one maternal psychiatric condition, maternal smoking in any month during preconception and pregnancy, and maternal age at birth	< 1 drink per week in month 1 or month 2 was not associated with ASD; 1-2 drinks in month 1 were associated with lower odds of ASD (1-2 drinks in month 2 not significant).

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/ Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Skogerbø et al. (2012)[31]	Danish National Birth Cohort Lifestyle During Pregnancy Study (*n* = 1,628)	Denmark 1997-2003	1 to 4 drinks per week	5 to 8 drinks per week	12 g pure alcohol	Interview at 17 weeks	Behavior Rating Inventory of Executive Function parent and teacher forms; age 5	Parental education, maternal IQ, prenatal maternal smoking, child's age at testing, child's gender, maternal binge drinking, maternal age, parity, maternal marital status, family home environment, postnatal parental smoking, pre-pregnancy maternal BMI, health status of child	No association existed between low or moderate PAE and executive function.
Skogerbø et al. (2013)[30]	Danish National Birth Cohort Lifestyle During Pregnancy Study (*n* = 1,628)	Denmark 1996-2002	1 to 4 drinks per week	N/A	12 g pure alcohol	Interview at 17 weeks	Parent- and teacher-rated SDQ, age 5	Maternal binge drinking, parental education, maternal IQ, prenatal maternal smoking, child's age at testing, child's gender, maternal age, parity, maternal marital status, family-home environment, postnatal parental smoking, pre-pregnancy maternal BMI, and child's health status	No association existed between low to moderate alcohol consumption and offspring behavior.
Underbjerg et al. (2012)[28]	Danish National Birth Cohort Lifestyle During Pregnancy Study (*n* = 1,628)	Denmark 1997-2003	1 to 4 drinks per week	5 to 8 drinks per week	12 g pure alcohol	Interview at 17 weeks	Test of Everyday Attention for Children at Five, age 5	Parental education, maternal IQ, maternal smoking in pregnancy, child's age at testing, gender, and tester were considered core confounding factors, whereas the full model also controlled the following potential confounding factors: maternal binge drinking or low to moderate alcohol consumption, age, BMI, parity, home environment, postnatal smoking in the home, child's health status, and indicators for hearing and vision impairments	No association existed between low or moderate PAE and attention.

Appendix 1. Studies of Low to Moderate Prenatal Alcohol Exposure and Neurodevelopmental Outcomes (Continued)

Author (Year)	Data Source (*n*)	Setting; Offspring Birth Years	Dose of PAE: Low/Light	Dose of PAE: Moderate	Definition of One Drink/Unit of Alcohol	PAE Measurement	Outcome; Offspring Age at Measurement	Covariates	Finding
Weile et al. (2020)[24]	Aarhus Birth Cohort (*n* = 48,072)	Denmark 1998-2012	Analyzed as categories: < 1, 1, 2, or ≥ 3 drinks per week	N/A	12 g pure alcohol	Questionnaire in early pregnancy (median 11 weeks)	ADHD diagnosis from Danish health registries, median age 12 (up to age 19)	Maternal age, highest attained educational level, chronic disease, presentational BMI, smoking in pregnancy, parity, birth year, binge drinking	Up to 1 drink per week PAE was associated with lower risk of ADHD; no associations existed with 2 or more drinks per week.
Williams Brown et al. (2010)[45]	Early Childhood Longitudinal Studies—Birth Cohort (*n* = 10,700)	United States 2001	Low to moderate: 0 to 4 drinks per week Analyzed in categories < 1 drink per week, 1-3 drinks per week		Not reported	Maternal report at 9 months postpartum (range 6 to 22 months)	Bayley Short Form—Research Edition; Nursing Child Assessment Teaching Scale, Behavior Rating Scale, Infant/Toddler Symptom Checklist; age 9 months	Race, poverty, child's age at assessment	< 1 drink or 1 to 3 drinks were not associated with most outcomes including Bayley Mental or Motor subscales, sensory regulation variables, and behavior rating scale. < 1 drink and 1 to 3 drinks were associated with undesirable social engagement and child interaction.

Note: AA, absolute alcohol; ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; BAS, British Ability Scale; BMI, body mass index; BSID, Bayley Scales of Infant Development; BSID-III, Bayley Scales of Infant and Toddler Development, third edition; CBCL, Child Behavior Checklist; dl, deciliter; g, grams; K6 score, Kessler Psychological Distress Scale score; K-SADS, Schedule for Affective Disorders and Schizophrenia for School-Age Children; MDI, mental development index; ml, milliliter; N/A, not applicable; NEPSY-II, NEuroPSYchological assessment, second edition; NIH, National Institutes of Health; PAE, prenatal alcohol exposure; PDI, psychomotor development index; *SD*, standard deviation; SDQ, Strength and Difficulties Questionnaire; SES, socioeconomic status; T1, first trimester; T2, second trimester; T3, third trimester.

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NIAAA 50th ANNIVERSARY FESTSCHRIFT

Fetal Alcohol Spectrum Disorders: Awareness to Insight in Just 50 Years

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This article is part of a Festschrift commemorating the 50th anniversary of the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Established in 1970, first as part of the National Institute of Mental Health and later as an independent institute of the National Institutes of Health, NIAAA today is the world's largest funding agency for alcohol research. In addition to its own intramural research program, NIAAA supports the entire spectrum of innovative basic, translational, and clinical research to advance the diagnosis, prevention, and treatment of alcohol use disorder and alcohol-related problems. To celebrate the anniversary, NIAAA hosted a 2-day symposium, "Alcohol Across the Lifespan: 50 Years of Evidence-Based Diagnosis, Prevention, and Treatment Research," devoted to key topics within the field of alcohol research. This article is based on Dr. Charness' presentation at the event. NIAAA Director George F. Koob, Ph.D., serves as editor of the Festschrift.

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The establishment of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) in 1971 was bracketed by three seminal papers that laid the groundwork for the field of fetal alcohol spectrum disorders (FASD) research. In 1968, Lemoine et al.[1] described children with birth defects and neurodevelopmental disorders associated with prenatal alcohol exposure (PAE). This French-language report was not widely appreciated until after the publication in 1973 of two landmark papers in *The Lancet*,[2,3] providing the first English-language description of fetal alcohol syndrome (FAS). The subsequent recognition of the high global prevalence of FASD and FAS highlighted a paradox. If alcohol and PAE have been ubiquitous since antiquity, why was FASD not recognized sooner?

Indeed, there were hints dating back to biblical times that PAE was harmful to the developing fetus (reviewed by Jones and Smith[2] and by Warren[4]). The London gin epidemic from 1690 to 1752 led to a petition by the London College of Physicians to the House of Commons to reimpose a tax on spirits, noting "Spirituous Liquors…[are] too often the cause of weak, feeble, and distempered children who must be instead of an advantage and strength a charge to their country."[4] Their petition implicated distilled spirits, rather than alcohol, per se, and did not impugn beer. Human and animal studies from the early 20th century suggested that PAE adversely affected pregnancy outcomes; however, when NIAAA was first established, the prevailing view was that alcohol was not harmful to the developing fetus, and high-dose, intravenous alcohol continued to be administered to some pregnant women to prevent premature labor. Thus, one of NIAAA's seminal accomplishments was the nurturing of FASD research and the deployment of research findings to alert clinicians, legislators, and the public to the dangers of PAE.

This brief review focuses on selected discoveries of the last half century on the effects of PAE, highlighting the work of NIAAA-funded researchers as well as the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD), a research consortium funded by NIAAA from 2003 until the present, for which Dr. Ed Riley has served as principal investigator and the author of this review has served as scientific director. Readers are referred to more comprehensive reviews of FASD for additional information.[5,6]

Fetal Alcohol Syndrome

The major functional disabilities associated with PAE are due to lifelong cognitive and behavioral impairment.[5] Alcohol affects brain development throughout pregnancy, yet the neuropathology is often microscopic and not evident on clinical imaging. What made FAS recognizable to early investigators was not a specific neurodevelopmental syndrome, but rather the associated constellation of prenatal and postnatal growth retardation, small head circumference (microcephaly), and facial and nonfacial dysmorphology in infants or children with PAE.[2,3] Microcephaly and prenatal and postnatal growth retardation are found in numerous neurodevelopmental disorders. However, alcohol exposure during one of the earliest embryonic developmental stages (i.e., gastrulation) induces relatively specific facial dysmorphology that serves as a visible marker for the underlying brain and neurodevelopmental abnormalities that cause functional impairment. This specific facial dysmorphology provided the long-missing link between PAE, abnormal brain development, and neurodevelopmental abnormalities. It is the frequent absence of this specific facial dysmorphology and the difficulty of obtaining a history of PAE that have challenged clinicians and investigators in fully characterizing the neurodevelopmental outcomes associated with alcohol exposure at other stages of gestation.

Diagnosis of FAS and FASD

There is no biological marker or gold standard that identifies a child with FASD. Consequently, as research on FASD progressed over the past half century, diagnostic criteria for FASD, including FAS, evolved and diverged, both within the United States and in other countries.[5,7-10]

All diagnostic systems for FAS require either two or three of three cardinal facial features: short palpebral fissures; smooth nasal philtrum; and thin upper lip vermilion. All diagnostic systems also require structural and/or functional abnormalities of the central nervous system. Prenatal and postnatal growth retardation, although predictive of adverse neurodevelopmental outcomes,[11] are not universally required for diagnosis. FAS may be diagnosed in the absence of a history of PAE, given the relative specificity of the cardinal facial features, particularly after ruling out phenocopies of FAS, including genetic conditions and other teratogenic exposures (see Table 4 in Hoyme et al. [2016][7]).

Absent a gold standard, no diagnostic system can be considered superior, and agreement among diagnostic systems within a single cohort is modest.[8,9] Clearly, clinical care and research on FASD would benefit from the harmonization of these various diagnostic and classification systems. Below is a more detailed description of one representative diagnostic framework, which was developed by the Collaboration on Fetal Alcohol Spectrum Disorders Prevalence (CoFASP), a study funded by NIAAA to investigate the epidemiology of FASD across the United States.[7] According to CoFASP, the umbrella term FASD encompasses any one of four conditions: FAS, partial fetal alcohol syndrome (PFAS), alcohol-related neurodevelopmental disorder (ARND), and alcohol-related birth defects (ARBD) (see Table 1).[7]

Table 1. Diagnostic Criteria for Four Conditions Within the FASD Spectrum According to CoFASP.[5]

Diagnostic Criterium	FAS		Partial FAS		ARND	ARBD
Confirmed Prenatal Alcohol Exposure[a]	Yes	No	Yes	No	Yes	Yes
Facial Dysmorphology[b]	Required	Required	Required	Required	Not required	N/A
Growth Deficiency[c]	Required	Required	Not required	Required if brain abnormality is not present	Not required	N/A
Brain Abnormality[d]	Required	Required	Not required	Required if growth deficiency is not present	Not required	N/A
Cognitive or Behavioral Impairment[e]	Required	Required	Required	Required	Required*	N/A
Other Systemic Malformation	Not required	Not required	Not required	Not required	Not required	Required

[a] Defined as ≥ 6 drinks/week for 2 weeks or ≥ 3 drinks on ≥ 2 occasions; documentation of maternal intoxication in records; positive biomarker for alcohol; or evidence of risky maternal drinking on a validated screening tool.

[b] Defined as ≥ 2 of the following: short palpebral fissures, thin vermilion border, and smooth philtrum.

[c] Defined as height and/or weight ≤ 10th centile based on racially/ethnically normed charts.

[d] Defined as head circumference ≤ 10th centile, structural brain anomaly, or recurrent nonfebrile seizures.

[e] Cognitive impairment is defined as global cognitive impairment, verbal or spatial IQ, or individual neurocognitive domain ≥ 1.5 SD below mean. Behavioral impairment is defined as impairment of self-regulation ≥ 1.5 SD below mean. For children under age 3, developmental delay is required.

* ARND requires two behavioral or cognitive deficits if IQ is not ≥ 1.5 SD below the mean.

Note: ARBD, alcohol-related birth defects; ARND, alcohol-related neurodevelopmental disorder; FAS, fetal alcohol syndrome; N/A, not applicable.

Source: Adapted with permission from Wozniak et al.[5]

The CoFASP diagnostic criteria for FAS require abnormalities in four clinical domains: craniofacial anomalies; growth retardation; abnormal brain structure or function; and neurobehavioral impairment.[7] Short palpebral fissures are identified when direct measures are in the 10th centile or below. Smooth philtrum and thin vermilion border are identified by comparing facial features to racially normed lip/philtrum charts. Only two of the three cardinal craniofacial anomalies need be present for an FAS diagnosis. Prenatal and/or postnatal growth deficiency is defined as height and/or weight in the 10th centile or below. Abnormal brain structure or function may include head circumference in the 10th centile or below, structural brain abnormalities, or recurrent nonfebrile seizures.

In the CoFASP framework, neurobehavioral impairment is measured using standardized tests and may include cognitive deficits, such as low full-scale IQ, as well as impairment of executive functioning, learning, memory, visuospatial perception, or behavior, including self-regulation, attention, and impulse control. Most of these impairments are defined based on scores of at least 1.5 standard deviation (SD) below the mean. For children younger than age 3, the criterion for neurobehavioral impairment is met if there is developmental delay of at least 1.5 SD below the mean.[7] Other diagnostic systems set different thresholds for dysmorphology and neurobehavioral impairment. For example, a framework included in the most recent edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5)[12] as a condition for further study focuses on three domains of impairment (i.e., neurocognitive, self-regulation, and adaptive function).[13] Mattson and colleagues provide a detailed description of neurobehavioral impairment in FASD.[14]

According to the CoFASP framework, with documented PAE, a diagnosis of PFAS is made when there is cardinal facial dysmorphology and neurobehavioral impairment but no growth retardation and no abnormal brain structure or function; absent evidence of PAE, the diagnosis of PFAS additionally requires growth deficiency or deficient brain growth.[7] ARND is diagnosed when there is neurobehavioral impairment and a history of PAE but no cardinal facial

dysmorphology.[7] Although many children with FASD have a constellation of dysmorphic features affecting the face, limbs, and internal organs, PAE in rare cases causes major malformations without neurobehavioral impairment, structural brain abnormalities, or growth retardation. This condition is referred to as ARBD.[7]

The definition of PAE assumes great importance in clinical diagnosis and research but differs among the different diagnostic systems. CoFASP defines PAE based on one of the followed criteria for maternal alcohol consumption: six or more drinks per week for two or more weeks during pregnancy; three or more drinks on at least two occasions; alcohol-related social or legal problems around the time of pregnancy; documented intoxication during pregnancy; positive testing for biomarkers associated with alcohol exposure; or a positive screen using a validated tool for alcohol use.[7]

Alcohol Is a Teratogen

Many children diagnosed with FASD have been exposed to other drugs, such as nicotine, cannabinoids, opioids, or stimulants; have nutritional deficiency; are raised in chaotic households; and experience numerous adverse childhood events. Separately, each of these insults may cause neurobehavioral impairment. Therefore, not surprisingly, there was initial reluctance to accept that alcohol causes birth defects (i.e., is a teratogen).

Animal models can control for many of these confounding variables and provided the first strong evidence that alcohol was indeed teratogenic. Sulik and colleagues showed that a single alcohol exposure during gastrulation in mice caused microcephaly, growth retardation, and the cardinal facial features of FAS in the absence of nutritional deficiency or other teratogens.[15] This discovery drew a direct connection between alcohol and the constellation of developmental abnormalities described less than a decade earlier in humans with FAS. It allowed the conclusion that alcohol toxicity causes FAS, even though concurrent teratogenic exposures, genetic polymorphisms, nutritional deficiency, and stressors may further impact craniofacial and brain development. Because gastrulation occurs during the third week of human gestation, when many women are unaware of their pregnancies, this seminal work also underscored the potential for binge drinking to cause FAS prior to pregnancy recognition.

Later work from the same laboratory highlighted both the relative specificity and insensitivity of the cardinal facial dysmorphology of FAS as a marker of PAE.[16,17] Whereas alcohol exposure in mice on gestational day 7 (corresponding to gastrulation) reproduced the cardinal facial dysmorphology of FAS, exposure on gestational day 8.5 (corresponding to neurulation) produced different facial anomalies more characteristic of DiGeorge syndrome or retinoic acid embryopathy. Indeed, retinoic acid exposure and alcohol exposure during gastrulation in mice caused similar malformations. Although alcohol and retinoic acid are chemically unrelated, their common potentiation of programmed cell death in selected embryonic cell populations induced similar, stage-dependent, developmental outcomes.[18,19]

These animal studies demonstrated that the presence and pattern of craniofacial malformations were dependent on the timing of teratogen exposure. The cardinal facial dysmorphology that was first and irrevocably associated with FAS proved to be a happenstance of alcohol exposure during gastrulation. These discoveries contributed to the recognition that, at least in some people, neurobehavioral impairment due to PAE could occur in the absence of cardinal facial dysmorphology or any facial dysmorphology at all, as is the case in people with ARND.

The Face Is a Window to the Brain

The first descriptions of FAS identified a variety of craniofacial abnormalities in addition to the cardinal features of short palpebral fissures, smooth nasal philtrum, and thin upper lip vermilion. Some of these malformations, such as maxillary hypoplasia, ptosis, and retrognathia, occur in a host of developmental disorders and are readily recognized by geneticists, dysmorphologists, and developmental pediatricians. Therefore, a major quest for the field has been the discovery of other patterns of facial or nonfacial dysmorphology that might also link neurobehavioral impairment to PAE, even in the absence of a history of PAE.

An equally important goal has been to simplify or automate the detection of any defining facial dysmorphology to facilitate diagnosis for the many patients that lack access to highly specialized clinicians. Astley used computer analysis of two-dimensional facial photographs to evaluate the diagnostic features defined by the FASD 4-Digit Diagnostic Code,[20] whereas CIFASD and other investigators employed automated analysis of three-dimensional (3D) facial images. Suttie and colleagues used dense surface modeling to study facial dysmorphology in 3D images of children from the CIFASD cohort.[21] This method allowed them to quantitate facial shape and to sort facial images based on the degree to which they resembled those of children with FAS or controls. This analysis differentiated children with PAE whose faces did not clearly show the characteristic features (i.e., those who had nonsyndromal faces) from children without PAE with greater than 90% specificity. Importantly, children with PAE who had nonsyndromal facial features also had significantly lower IQ and learning ability than children whose faces more closely resembled controls. Using dense surface modeling,

Muggli and colleagues demonstrated that even mild PAE could affect facial shape.[22]

Technology has evolved to enable the acquisition of 3D images on smartphones, and contour analysis can be automated in the Cloud. Hence, it may be possible to automate the analysis of facial dysmorphology and facilitate the diagnosis of FASD wherever access to internet-connected smartphones is available.

Epidemiology of FASD

FASD is the most common preventable cause of intellectual disability.[23] Using active case ascertainment, CoFASP investigators estimated the prevalence of FASD among first-grade students to be 1% to 5% across four regions of the United States.[24] These conservative estimates of FASD prevalence equal or exceed those for autism spectrum disorder. Among 222 cases identified as FASD within this cohort, 12% were classified as FAS, 47% as PFAS, and 41% as ARND. However, only two of the 222 children (1%) had previously been diagnosed with FASD, highlighting the extent to which FASD is underrecognized or misdiagnosed.[25]

Estimates of FASD prevalence vary across studies, in part because of differences in study methodology and classification definitions. One meta-analysis estimated the global prevalence of FAS at 0.15% and FASD at 0.77%.[26] Prevalence estimates also vary across different countries due to cultural differences in drinking. One of the highest estimates of FASD prevalence has been 14% to 21% in the wine-growing region of the Western Cape Province of South Africa, where weekend binge drinking has been common.[27]

High rates of binge drinking during the childbearing years are an important contributor to the high prevalence of FASD in the United States. Approximately 25% of Americans ages 18 to 44 binge drink, 45% of pregnancies are unintended, and gastrulation often occurs before a woman is aware of her pregnancy.[28,29] Among pregnant women, the prevalence of any alcohol use (10%) and binge drinking (3%) within the past 30 days is also high. The combination of binge drinking and sex without contraception greatly increases the risk of an alcohol-exposed pregnancy.

Whereas binge drinking is a widely accepted risk for FASD, there is less certainty regarding the risk associated with low or moderate levels of alcohol consumption during pregnancy, stemming in part from the inherent challenge of proving safety as opposed to harm. Both human and animal studies have failed to establish a threshold for safe drinking during pregnancy.[30] For example, in cell culture experiments, alcohol concentrations corresponding to those achieved in the blood and fetus after just one drink inhibit cell adhesion mediated by the developmentally critical L1 neural cell adhesion molecule.[31] In humans, intake of less than five to six standard U.S. drinks per week is associated with craniofacial dysmorphology and neurobehavioral impairment.[22,30,32] Research funded by NIAAA has played a major role in informing the advisories from the U.S. Surgeon General that women who are pregnant or trying to conceive should not consume alcoholic beverages.[4]

The Neurodevelopmental Effects of PAE

Early autopsy studies in infants and children with FAS revealed major brain malformations.[33] Among these were microcephaly, agenesis or hypoplasia of the corpus callosum, ventricular enlargement, dysplasia of the anterior lobes of the cerebellum, and neuronoglial heterotopias—findings consistent with major disruption of neurogenesis, neural cell migration, and the premature triggering of programmed cell death. These gross neuropathological abnormalities are not observed in most children with FASD but highlight the mechanisms underlying similar, but milder, abnormalities in grey matter thickness, microstructural white matter abnormalities, decreased brain volume, and neuronal and glial migration defects.[5] Prenatal alcohol exposure also alters the trajectory of grey matter development during childhood.[34] In some studies, facial abnormalities correlated with volume reductions in specific brain regions, reinforcing the concept that face and brain dysmorphology arise concurrently and that the face is a window to the brain.[17,35,36] Clinical imaging in children with FASD is frequently normal, reflecting the microscopic nature of brain developmental abnormalities that underlie typical neurobehavioral impairments related to PAE. Overall, studies found that neurodevelopmental outcomes are related to the quantity, frequency, and timing of alcohol exposure as well as to maternal age, nutritional status, socioeconomic status, and genetic background of both mother and fetus.[5]

Animal studies have shown that alcohol disrupts brain development through a variety of mechanisms. Alcohol causes oxidative injury and programmed cell death in neural crest cells destined to form craniofacial and brain structures.[15,18,37] Alcohol is metabolized to acetaldehyde, a toxic molecule that chemically modifies and damages DNA and cells.[38] Alcohol also produces enduring epigenetic changes[39] that alter DNA transcription and diverse signaling pathways involved in brain development. Moreover, alcohol impairs neurogenesis and diverts differentiation of neural stem cells from neural to nonneural lineages, contributing to brain volume reductions.[38] Early research further identified similarities between FAS and milder phenotypes of syndromes associated with holoprosencephaly,[40] a disorder that affects midline craniofacial and brain

development and is sometimes associated with mutations in the Sonic hedgehog (Shh) gene. Alcohol similarly disrupts the Shh signaling pathway,[41,42] thereby altering the function of primary cilia[43]—cellular organelles that are critical for development.

Alcohol also may disrupt neuronal cell migration and synaptic connections through its interactions with the L1 protein, a developmentally critical neural cell adhesion molecule that guides neuronal cell migration and axon pathfinding. Alcohol inhibits L1-mediated cell adhesion at half maximal concentrations achieved after just one drink.[31] Alcohol blocks L1 adhesion by binding to specific amino acids that regulate the interaction of L1 molecules located on adjacent cells.[44] The nanopeptide NAPVSIPQ potently antagonizes alcohol inhibition of L1 adhesion and prevents alcohol teratogenesis in mouse embryos.[45]

Finally, genetic factors also may influence the development of FAS and alcohol's effects on neurodevelopment. Concordance for FAS is higher in monozygotic than dizygotic twins,[46] and diverse genes have been identified that modulate the effects of alcohol on craniofacial and brain development.[47,48]

Biomarkers of Alcohol Exposure and Adverse Outcome Risk

In many cases, information on an infant's history of PAE is unavailable or unreliable, hampering the clinical diagnosis of FASD and related research. Analyses of early markers of alcohol exposure, such as fatty acid ethyl esters in meconium, can provide relatively sensitive and specific confirmation of PAE in the last two trimesters of pregnancy[49] but are not routinely performed in clinical practice. More recent research from CIFASD has raised hopes that biomarkers of exposure and risk for adverse outcomes may be obtained during the second trimester to identify infants and children requiring early intervention. For example, maternal blood samples from the second trimester of pregnancy showed increased methylation of pro-opiomelanocortin and period 2 genes,[50] unique cytokine signatures,[51] and a unique profile of micro RNAs linked to alcohol exposure and neurodevelopmental delay.[52] Infant plasma micro RNAs also predicted PAE-associated growth restriction and cognitive development.[53]

Some of these biomarkers also may be mediators of biological effects of PAE. Decreased expression of pro-opiomelanocortin was associated with increased levels of cortisol in children with PAE, consistent with disinhibition of the hypothalamic pituitary stress axis.[50] The identified micro RNAs were shown to collectively modulate placental growth and development,[54] and proinflammatory cytokines may predispose to autoimmune and inflammatory conditions later in life.[55,56]

FASD Across the Lifespan

The effects of PAE on morphology and neurobehavior and health are lifelong.[5] As children with FASD mature into adulthood, the cardinal facial dysmorphology may become less pronounced, making diagnosis in adulthood more difficult.[57] More challenging still is the diagnosis of FASD in adults with neurobehavioral disorders who lack both cardinal facial dysmorphology and a history of PAE. The high prevalence of FASD makes it likely that many such individuals are followed in adult medical practices without ever being diagnosed.

A growing area of FASD research concerns the developmental origins of health and disease associated with PAE.[58] Premature death and increased prevalence of metabolic, immune, and cardiovascular disorders have been reported in informal surveys of adults with FASD[56] as well as in epidemiological studies.[55,59] For example, studies in human cohorts and zebrafish indicate that PAE induces elements of metabolic syndrome in adults by modifying developmental programs for hepatic and adipose tissue embryogenesis.[60] Further research will be important to delineate the full range of human diseases associated with PAE to allow for earlier detection and intervention.

The Next 50 Years

What should we hope for from the next 50 years of NIAAA-funded research on FASD? There will never be enough specialized clinics to diagnose and treat the large numbers of children and adults with FASD. Recent advances in remote diagnosis of facial dysmorphology and in neurobehavioral assessment[61] hold promise for broader access to automated, cloud-based screening and diagnostic tools. The identification of more specific markers of PAE and adverse developmental outcomes will greatly aid diagnosis. Treatment is similarly limited by the high prevalence of FASD in relation to the availability of skilled therapists. The refinement of early interventions and their translation to accessible online platforms will be necessary to fully address the public health burden of FASD. App-based approaches show early promise but still require considerable development and refinement.[62] Studies on the postnatal administration of choline to mitigate the neurodevelopmental effects of PAE also have been encouraging.[63-65] Finally, the high prevalence of FASD will most readily be reduced by continued progress in one of NIAAA's primary missions—the development of effective strategies to prevent and treat alcohol use disorder and the patterns of drinking that engender PAE. Equally important will be the reduction in stigma associated with these disorders, so that effective strategies are embraced by those at risk or affected.

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Maternal Substance Use: Consequences, Identification, and Interventions

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Alcohol, tobacco, and cannabis are the substances most frequently used during pregnancy, and opioid-exposed pregnancies have increased fourfold. The purpose of this review is to describe the prevalence and consequences of prenatal exposure to alcohol, tobacco, cannabis, and opioids. Currently available screening questionnaires for prenatal substance use are summarized and contrasted with the measures available for prenatal alcohol use. Because screening for prenatal alcohol and substance use is but the prelude to efforts to mitigate the potential adverse consequences, attempts for the modification of these consequences are briefly reviewed. In addition, areas of future research related to the criminalization of prenatal substance use, which may inhibit both inquiry and disclosure, are discussed. Indeed, the full potential of effective interventions has yet to be realized.

KEY WORDS: prenatal alcohol substance use; screening and intervention

INTRODUCTION

Prenatal exposure to alcohol and other substances has become increasingly common. The substances used most frequently during pregnancy are alcohol, tobacco, and cannabis. Moreover, between 1999 and 2014, the number of women with opioid use disorder during labor and delivery quadrupled.[1] The purpose of this review is to describe the prevalence and consequences of prenatal exposure to alcohol, tobacco, cannabis, and opioids. Currently available screening questionnaires for prenatal substance use are summarized and contrasted with the measures available for prenatal alcohol use. Because screening for prenatal alcohol and substance use is but the prelude to efforts to mitigate the potential adverse consequences, attempts for the modification of these consequences are also briefly reviewed.

It should be noted that this review article is not intended to be a systematic review of the world literature on either prenatal substance use or its prevention. Rather, it is a narrative literature review

that is meant to be illustrative and to stimulate areas of future research because the full potential of effective interventions has yet to be realized.

THE CONSEQUENCES OF PRENATAL SUBSTANCE USE

The consequences of prenatal substance use differ depending on the specific substances used. The most commonly used substances include alcohol, tobacco, cannabis, and opioids.

Prenatal Alcohol Use and Its Consequences

The estimated percentage of prenatal alcohol use is approximately 15%, with past month use being approximately 13%.[2,3] A Centers for Disease Control and Prevention survey conducted from 2015 to 2017 found that nearly 4% of pregnant women had engaged in binge drinking in the prior 30 days.[4] Alcohol use during pregnancy is a highly preventable cause of birth defects and developmental disabilities.[5] Despite the recognition of the teratogenic properties of alcohol, many women continue to disregard advisories on avoiding alcohol during pregnancy.[6]

There is no known safe level of alcohol use while pregnant because there is no exact dose-response relationship between the amount of alcohol consumed during the prenatal period and the extent of damage caused by alcohol in the fetus.[7] Thus, an infant born to a mother who drank alcohol while pregnant may be normal or may manifest alcohol-related birth defects (e.g., problems with the heart, kidneys, bones, or hearing), alcohol-related neurodevelopmental disorders (e.g., intellectual disabilities or problems with behavior and learning), or fetal alcohol spectrum disorders (FASD), which includes a wide range of effects, from mild to severe. An individual with FASD might have abnormal facial features; small head size; shorter than average height; low body weight; poor coordination; hyperactive behavior; difficulty with attention; poor memory; difficulties in school, especially with mathematics; learning disabilities; speech and language delays; intellectual disability or low IQ; poor reasoning and judgment skills; sleep and sucking problems as a baby; vision or hearing problems; and problems with the heart, kidneys, or bones.[8]

A recent multisite study using active case ascertainment methods estimated that the prevalence of FASD among first graders ranged from 1% to 5%.[9] This is concerning because these disorders are associated with life-long disabilities. However, early intervention treatment services can improve a child's development and function.[8]

There is continuing uncertainty about the effects of low and low-to-moderate levels of alcohol intake during pregnancy.[10] For example, a recent cohort study reported craniofacial changes with almost any level of prenatal alcohol intake, but the clinical significance of these changes is not known.[11] Factors that may influence the effects of prenatal alcohol use include patterns of maternal drinking, maternal and fetal genetics, as well as socioeconomic and ethnic factors. Because there is no proven "safe" level of alcohol exposure during pregnancy, the most prudent advice for pregnant women is to abstain from drinking.[12]

Prenatal Tobacco Use and Its Consequences

Cigarette smoking in the antepartum period is common. Past month use of tobacco products among pregnant women was approximately 15% according to the 2017 National Survey on Drug Use and Health report.[13] Tobacco products include the use of alternative forms of nicotine, such as e-cigarettes and vaping, which until recently, have been perceived to be less harmful. For example, in 2015, as many as 7% of women with a recent live birth in Oklahoma and Texas reported using an electronic vapor product shortly before, during, or after pregnancy.[14] Data specific to the effects of prenatal use of electronic vapor products are sparse. However, the Centers for Disease Control and Prevention has issued interim guidance that electronic cigarette products should never be used by pregnant women or adults who do not currently use tobacco products as it investigates

the more than 200 cases of severe pulmonary disease associated with their use.[15]

The use of any tobacco product during pregnancy is associated with adverse maternal, fetal, and neonatal outcomes. Examples of the adverse consequences of tobacco use may begin with subfertility and delay in conception among women who smoke and extend to pregnancy outcomes, which include increased risk of spontaneous pregnancy loss, placental abruption, preterm premature rupture of membranes, placenta previa, preterm labor and delivery, low birth weight, and ectopic pregnancy. Prenatal cigarette smoking may exert effects beyond pregnancy as well and is associated with increased risks of asthma, infantile colic, and childhood obesity.[16]

Prenatal Cannabis Use and Its Consequences

Past month cannabis use among pregnant women ages 18 to 44 increased between 2002 and 2017 from approximately 3% to 7%.[17] Among pregnant adolescents, past month use (15%) was even higher.[18] A recent cross-sectional study using data from 367,403 pregnancies among 276,991 women in Northern California found that self-reported daily, weekly, and monthly cannabis use before and during pregnancy increased between 2009 and 2017. The greatest increases were for daily use, reaching 25% among those who used in the year before pregnancy and 21% among those who used during pregnancy.[19] Explanations for the increases in prenatal use include increasing acceptance of cannabis use and decreasing perceptions of cannabis-related harms.[20]

The association between prenatal cannabis use and maternal, perinatal, and neonatal outcomes is unclear.[21] A 2016 systematic review and meta-analysis concluded that maternal marijuana use during pregnancy was not an independent risk factor for adverse neonatal outcomes, such as low birth weight or preterm delivery, after adjusting for confounding factors like tobacco use.[22] However, limitations to the generalizability of this meta-analysis include the relatively few women in the risk-adjusted group, indicating that the meta-analysis was underpowered to stratify for all secondary outcomes of interest. Another systematic review and meta-analysis from the same time frame found that pregnant women who used marijuana had increased odds of being anemic and that infants exposed to cannabis in utero had decreased birth weight and were more likely to require neonatal intensive care.[23] The researchers from this review acknowledged that because many cannabis users often use tobacco and alcohol as well, discerning a cannabis-only effect was not possible. A population-based cohort study of 661,617 women in Ontario, Canada, showed that the percentage of preterm births among self-reported cannabis users was 12% compared to 6% among nonusers, with this increase persisting even after adjusting for confounding factors.[24] Until there is definitive evidence demonstrating the safety of prenatal marijuana use, concerns that marijuana may interfere with neurodevelopment as well as have other effects have resulted in the American College of Obstetricians and Gynecologists (ACOG) advising women who are pregnant or thinking about pregnancy to avoid using marijuana and other cannabinoids.[25]

Prenatal Opioid Use and Its Consequences

Opioid use among pregnant women increased fourfold between 1999 and 2014 and is present in approximately 3% of pregnancies.[26] Women who use opioids during pregnancy are a diverse group because opioid use may occur in the context of medical care, opioid misuse, or untreated opioid use disorder.[27]

Prenatal opioid use can have a far-reaching clinical impact on infant outcomes. Infants with prenatal opioid exposure are typically born smaller and may have neonatal opioid withdrawal syndrome (NOWS). Infants with NOWS experience withdrawal from opioids and require additional medical care.[28] Characteristics of NOWS, also known as neonatal abstinence syndrome (NAS), include disturbances in gastrointestinal, autonomic, and central nervous systems, leading to irritability,

high-pitched crying, poor sleep, and uncoordinated sucking reflexes that lead to poor feeding. In 2014, a baby was born with NOWS in the United States every 15 minutes.[29,30]

The full impact of opioid exposure during pregnancy on fetal, infant, and childhood outcomes, however, is still unknown. Explanations include the possibility of exposure to other substances as well as concomitant maternal, medical, psychological, and socioeconomic issues. There is a growing body of evidence about the association of opioids with specific birth defects, such as congenital heart defects, neural tube defects, and clubfoot.[31]

For pregnant women with opioid use disorder, substitution treatment with opioid agonists, such as methadone and buprenorphine, imparts important benefits particularly when compared to continued illicit drug use. Advantages include more stable maternal drug levels, reduced withdrawal and drug-seeking behavior, and improved self-care, which should lead to a better pregnancy outcome because of reduced risk for fetal distress, miscarriage, growth restriction, and preterm birth.[32]

Compared to data on buprenorphine-maintained pregnancies, more longitudinal data on methadone-exposed pregnancies are available. In a prospective longitudinal study, 68 methadone-exposed children and 88 nonmethadone-exposed children were evaluated at 2.0 and 4.5 years for executive functioning and later emotional behavioral and emotional adjustment.[33] The methadone-exposed children had worse inhibitory control than the nonexposed children, when taking maternal education and prenatal benzodiazepine use into account. Another study used a school readiness framework to assess the health and neurodevelopmental outcomes of a regional cohort of 100 methadone-exposed children and 110 randomly identified nonmethadone-exposed children who were studied from birth to 4.5 years. Children born to opioid-dependent mothers had higher rates of delay and impairment across all outcome domains, with multiple domain problems being common. Impaired school readiness was associated with greater maternal substance use, higher social risk, male sex, and lower quality caregiving environments.[34]

A systematic review and meta-analysis synthesized data from 41 studies on the neurodevelopment of prenatal methadone-exposed children. The analysis included 1,441 children whose mothers were prescribed methadone during pregnancy and 842 children whose mothers did not receive methadone.[25] Methadone-exposed children appeared to be at increased risk for neurodevelopmental impairment, with lower scores on the Mental Development Index and Psychomotor Development Index, as well as atypical visual evoked potentials, strabismus, and nystagmus. However, these findings about impairment may be biased, with the studies not accounting for factors other than methadone. Indeed, results from this meta-analysis confirm the need for more research and the many factors that can impact pregnancy outcome.

SCREENING FOR PRENATAL SUBSTANCE USE

Early universal screening of pregnant women for alcohol use, substance use, or both is recommended by ACOG because alcohol and substance use is not typically disclosed spontaneously by patients. ACOG recommends clinicians use validated questionnaires or have a conversation with patients but does not endorse using routine urine toxicology tests.[35,36] Moreover, a positive screening questionnaire does not result in a diagnosis. Rather, such a result is an opportunity for a patient and her clinician to review health practices and make changes, if appropriate.[37]

Screening for Prenatal Alcohol Use

There is no known safe level of alcohol consumption during pregnancy.[38] Alcohol is a teratogen; in other words, it is capable of interfering with fetal development, resulting in birth defects. Although the consequences of light alcohol use among women, defined as consuming up to 32 g of alcohol per week, on pregnancy outcomes remain unsettled in the absence of

sufficient evidence, the potential for harm cannot be ruled out.[12] Hence, ACOG has recommended that all women seeking obstetric–gynecologic care be screened for alcohol use annually and within the first trimester of pregnancy.

Screening questionnaires for prenatal alcohol use have been well studied. For example, a systematic review of brief screening questionnaires to identify problem drinking during pregnancy evaluated seven instruments given to 6,724 participants.[39] The measures included the TWEAK (Tolerance, Worried, Eye-Opener, Amnesia, K/Cut Down); the T-ACE (Tolerance [number of drinks], Annoyance, Cut Down, Eye-Opener); CAGE (Cut Down, Annoyed, Guilty, Eye-Opener), NET (Normal Drinker, Eye-Opener, Tolerance); AUDIT (Alcohol Use Disorder Identification Test); AUDIT-C (AUDIT Alcohol Consumption Questions), and SMAST (Short Michigan Alcoholism Screening Test). The screening questionnaires were compared with a structured interview to ascertain drinking status as a reference standard. The T-ACE, AUDIT-C, and TWEAK were the three questionnaires identified to be the most promising screening tools for identifying risk drinking in pregnant women. However, the sensitivity and specificity of these three questionnaires outside the United States is unknown.

Screening for Prenatal Substance Use

Screening instruments for prenatal alcohol use have been well studied, whereas screening instruments for substances other than alcohol have been less well developed.[26,40] The World Health Organization (WHO) guidelines for the identification and management of substance use and substance use disorder during pregnancy list the Substance Use Risk Profile-Pregnancy (SURP-P) scale,[41] the proprietary 4P's Plus©,[42] and the National Institute on Drug Abuse (NIDA) Quick Screen–Modified Alcohol, Smoking, and Substance Involvement Screening Test (ASSIST)[43] as potential screening measures for pregnant women, even though not all of these instruments had been evaluated among that population at the time of its recommendation.[44]

Several recent studies have evaluated the accuracy of various screening tools for prenatal substance use. In one prospective cross-sectional study conducted in Baltimore, MD, with 500 pregnant women, stratified by trimester and use of prenatal care, researchers administered three index tests and compared them to reference tests.[45] The three index tests were the proprietary 4P's Plus©, NIDA Quick Screen–ASSIST), and the SURP-P. The reference tests were urine and hair testing, which captured substance use up to the past 90 days. Alcohol use was not evaluated. The researchers found that there were differences in validity indices (i.e., sensitivity, specificity, positive predictive value, and negative predictive value) by age and race, but not by trimester, for all screening tools. The SURP-P and 4P's Plus© were highly sensitive across all trimesters, races, and age groups.

Another prospective cross-sectional screening accuracy study compared five screening instruments on their ability to identify illicit drug, opioid, and alcohol use under privacy expectations consistent with current practice. The participants included 1,220 pregnant women who were receiving care in Boston, MA; Detroit, MI; or New Haven, CT. The women were socioeconomically diverse and had a mean age of 29 years. The study used a reference standard of substance use in three classes (i.e., illicit drugs, opioids, and alcohol); results were considered positive if use was evident via a 30-day calendar recall or urine toxicology analysis.[46] The illicit drug use reference standard included marijuana, cocaine, heroin, amphetamines, barbiturates, and hallucinogens. The five screening instruments for substance use in pregnancy were the SURP-P; CRAFFT, a five-item screener with items related to car, relax, alone, forget, friends, and trouble; 5Ps, with items on parents, peers, partner, pregnancy, past (i.e., an adaptation of the 4P's Plus©); Wayne Indirect Drug Use Screener (WIDUS); and NIDA Quick Screen–ASSIST. None of the five measures showed both high sensitivity and high specificity, and the area under the curve was low for nearly all measures,

indicating that none could be recommended for applied practice with pregnant women.

A companion study compared the same five measures in the identification of substance use disorder, including alcohol, cannabis, opioids, and stimulants, among the 1,220 pregnant women.[47] Participants completed the Mini International Neuropsychiatric Interview 7.0.2, a short, structured diagnostic interview to identify substance use disorder, including alcohol; cannabis; stimulants, such as cocaine or amphetamines; and opioids, such as heroin and the nonmedical use of prescription drugs.[48] Substance use disorder is distinct from substance use and represents a more significant and persistent pattern of consumption that may increase the risk of adverse infant outcomes as well as indicate that the pregnant woman may need evaluation and referral for specialty treatment.[49] Of the 1,220 women in this study, more than 15% satisfied diagnostic criteria for substance use disorder and more than 30% reported having used alcohol or other substances in the past month. There was little overlap between the women who had substance use disorder and the women who had used alcohol or other substances within the past month. Nearly 10% of the women satisfied criteria for alcohol use disorder, as defined in the fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders*, and 9.0% satisfied criteria for substance use disorder. Specifically, cannabis use disorder was the most common substance disorder diagnosed (8%). Approximately 3% satisfied criteria for more than one disorder.

There were considerable variations by site. For example, alcohol use disorder was the most common in Boston (15%) but infrequent in New Haven (5%). In contrast, substance use disorder was the most common in Detroit (17%) but less frequent in Boston (3%). Measures of merit (i.e., sensitivity, specificity, accuracy, and area under the receiver operating curve [AUROC]) were calculated with 95% confidence intervals [CI] for the NIDA Quick Screen, CRAFFT, SURP-P, WIDUS, and 5Ps, using substance use disorder as the criterion standard. The CRAFFT (AUROC=0.75, 95% CI [0.72, 0.79]) and SURP-P (AUROC=0.74, 95% CI [0.71, 0.78]) had the highest AUROCs for identifying substance use disorder, including alcohol. In contrast, the NIDA Quick Screen had the lowest AUROC (AUROC=0.62, 95% CI [0.59, 0.65]) for identifying substance use disorder, including alcohol. Overall, the tested measures were more accurate in identifying alcohol use disorder than substance use disorder (e.g., for identifying alcohol use disorder, the AUROCs for the CRAFFT and SURP-P were 0.78 and 0.77, respectively).

Barriers to Early Identification by Screening

Pregnant women with substance use disorder are at increased risk for adverse health and social outcomes, making early identification crucial.[50] Because substance use is substantially underreported, even among women who participate regularly in urine drug screens, use of validated questionnaires to identify prenatal alcohol and substance use has been recommended.[26,51]

There are, however, at least two barriers to these recommendations. First, as discussed in the preceding section, current screening questionnaires have been found to be inadequate measures. According to a 2010 survey of obstetrician-gynecologists, 58% did not use a validated screening tool to assess alcohol risk despite there being several validated tools available.[52] It is likely that even fewer will use a screening tool for prenatal substance use, particularly as such tools are less well developed. A second barrier includes the punitive consequences stemming from state laws regarding prenatal substance use, which can result in patients not wanting to disclose and physicians not wanting to learn about their patients' behaviors.[53-55] Hence, in addition to patients' previous fears about stigmatization because of use, disclosure could now pose a legal risk.[56] An example of a punitive policy includes treating substance use during pregnancy as child abuse or neglect. This policy may arise from a desire to discourage women from using substances while pregnant, to encourage

women to seek treatment, and to ensure the safety of the neonate.[57]

The association between states with punitive or reporting policies related to substance use in pregnancy and rates of NAS was recently evaluated in a study of 4,567,963 births from 8 U.S. states in varying years between 2003 and 2014.[57] States without punitive or reporting policies were compared with states that had such policies, before and after policy enactment. The main outcome measure was the rate of NAS. States that criminalized substance use during pregnancy (e.g., grounds for civil commitment, child abuse, or neglect) had significantly higher rates of NAS in the 1st full year after enactment and more than 1 full year after enactment. In contrast, there was no association with neonatal abstinence rates in states with policies requiring reporting of suspected prenatal substance use. A possible explanation for this difference includes the extent to which pregnant women disengage from health care services when punitive measures are enforced, whereas reporting policies may not dissuade pregnant women from engaging with health care services, resulting in greater conversations between physicians and their patients. However, neither the punitive nor the reporting approach resulted in reduced rates of NAS, which was the presumed, desired outcome of these policies.

AFTER SCREENING: INTERVENTION

Because screening for prenatal alcohol and substance use is but the prelude to efforts to mitigate the potential adverse consequences, brief intervention and referral to treatment, if indicated, have also been recommended.[56] Brief interventions and psychosocial interventions have been examined by investigators and organizations such as the WHO, which sought to develop evidence-based global guidelines for identifying and managing substance use and substance use disorder in pregnancy.[42] Global guidelines were desired because although several high-income countries had developed national guidelines, low- and middle-income countries had not. However, the WHO noted that much of the evidence underlying the effectiveness of screening and brief interventions during pregnancy originated from a time when reporting standards and measures of bias were not in consistent use. Nonetheless, the evidence indicated that asking women about alcohol and other substance use in a detailed and comprehensive way may increase their awareness of the risks associated with these practices and prompt them to modify their behavior.

Psychosocial Interventions for Prenatal Alcohol Use

In late 2018, the U.S. Preventive Services Task Force (USPSTF) renewed its recommendation for screening adults ages 18 year or older, including pregnant women, for unhealthy alcohol use and providing persons engaged in risky or hazardous drinking with brief behavioral counseling interventions to reduce unhealthy alcohol use (i.e., a grade B recommendation meaning that there is high certainty that the net benefit is moderate, or moderate certainty that the net benefit is moderate to substantial).[56] The USPSTF bounds the harms of screening and brief behavioral counseling interventions for unhealthy alcohol use in adults as small to none, based on the likely minimal risks of completing screening questionnaires, the noninvasive nature of the interventions, and the absence of reported harms in the evidence of the behavioral interventions.

The USPSTF makes three special comments with regards to pregnant women. First, any alcohol use by pregnant women is unhealthy. Second, validated alcohol screening tools for pregnant women are available, including the T-ACE and TWEAK. Third, brief counseling interventions among pregnant women have increased the likelihood that women remain abstinent from alcohol use during pregnancy.

Most interventions for FASD have been reported in North America, which has lower FASD prevalence compared to Europe and other sites around the world.[57] Context-related differences may impact on the effectiveness of

the interventions. For example, in a systematic review of prevention interventions to reduce prenatal alcohol exposure and FASD in indigenous communities, reviewers evaluated studies conducted from 1989 to 2017. A total of 10 studies from an initial sample of 712 articles were included if inclusion criteria were met. Comparisons of study effects were made difficult by heterogenous study designs, target populations, and interventions. The reviewers concluded that there was minimal evidence to support the belief that interventions intended to reduce the risk of prenatal alcohol exposure or FASD in indigenous populations have been effective.[58]

Psychosocial Interventions for Prenatal Cigarette Smoking

Psychosocial interventions for supporting women to stop smoking during pregnancy were assessed by the Cochrane Pregnancy and Childbirth Group.[59] This review included 102 randomized controlled trials, with 120 intervention arms. Data from 88 randomized controlled trials, involving more than 28,000 women, were analyzed. Intervention strategies included counseling, health education, feedback, incentives, social support, and exercise. Nearly all studies were conducted in high-income countries. Results from the review yielded moderate- to high-quality evidence that psychosocial interventions increased the proportion of pregnant women who had stopped smoking by late pregnancy (35%), with a 17% reduction in infants born with low birth weight, and a 22% reduction in neonatal intensive care admissions. There did not appear to be any adverse psychological effects from the interventions.

Psychosocial Interventions to Reduce Other Prenatal Substance Use

Screening, brief intervention, and referral to treatment in the perinatal period have been recommended for prenatal substance use.[60] Subsequent to this recommendation, at least two systematic reviews of the evidence for psychosocial interventions have been completed.

The first systematic review included four articles published between 2002 and 2013. It began with 3,792 unique potential publications, but the vast majority did not meet a priori quality criteria. Limited, but promising, evidence of brief interventions reducing illicit drug use among postpartum women was found.[61]

The second systematic review was completed by researchers from the Cochrane Collaboration. They sought to evaluate the evidence on the effect of psychosocial interventions, such as contingency management (CM) and motivational interviewing-based (MIB) techniques compared to that of usual care for pregnant women in outpatient illicit drug treatment programs.[62] This group reviewed 14 studies, with 1,298 pregnant women who received either CM or MIB techniques in addition to other comprehensive care. The women in the control group received usual care that included pharmacological management, counseling, prenatal care, transportation, and/or childcare. There were no differences in retention or abstinence behavior between CM/MIB techniques and usual comprehensive care. The quality of evidence from these studies was assessed to be low to moderate.

SUMMARY

Prenatal exposure to alcohol, tobacco, and marijuana has become increasingly common. In addition, there has been a fourfold increase in the number of opioid-exposed pregnancies. Prenatal exposure to alcohol and other substances may have an adverse impact on a developing fetus. Since pregnant women may be reluctant to disclose their use or may not appreciate the potential for harm, early identification is desirable. However, identification is currently limited by the lack of adequate screening tools and the fear of legal and other sanctions, which may limit both inquiry and disclosure. Although effective interventions for prenatal alcohol, cigarette, and other substances are available, these interventions rely on identification and behavioral counseling. It is likely that the full potential of effective interventions cannot yet be realized in the current setting.

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Prenatal Alcohol Exposure and the Developing Immune System

Theresa W. Gauthier, M.D.

Evidence from research in humans and animals suggest that ingesting alcohol during pregnancy can disrupt the fetal immune system and result in an increased risk of infections and disease in newborns that may persist throughout life. Alcohol may have indirect effects on the immune system by increasing the risk of premature birth, which itself is a risk factor for immune-related problems. Animal studies suggest that alcohol exposure directly disrupts the developing immune system. A comprehensive knowledge of the mechanisms underlying alcohol's effects on the developing immune system only will become clear once researchers establish improved methods for identifying newborns exposed to alcohol in utero.

Key words: Alcohol in utero; prenatal alcohol exposure; fetal alcohol effects; alcohol-related intrauterine disorder; fetal alcohol syndrome; fetal alcohol spectrum disorders; immune system; immune function; fetal development; prenatal development; pregnancy; premature birth

Most Americans are aware that drinking alcohol during pregnancy can injure the developing fetus. Fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorders (FASD), with their developmental, cognitive, and behavioral consequences, probably are the best known dangers (Bakoyiannis et al. 2014; Centers for Disease Control and Prevention [CDC] 2009). However, drinking during pregnancy also can disrupt other areas of fetal development besides the brain, including the developing immune system. Studies in humans and animals suggest that alcohol does, in fact, affect the developing immune system and leads to increased risk of infection and disease in infants exposed to alcohol in utero.

Alcohol's effect on the developing immune system is apparent in infants born at term gestation, with studies showing that these babies are at increased risk of infection when exposed to alcohol in utero. However, premature infants are at even higher risk of infection for multiple reasons. For one, in utero alcohol exposure is associated with premature birth, which independently increases immune-related risks. In addition, animal studies show that alcohol has a direct effect on specific aspects of immune function, particularly in the developing lung. This article will discuss the short and long-term effects of drinking during pregnancy on the immune system of the developing fetus (see the figure for an overview).

Understanding the full extent of alcohol's threat to the developing fetus is critical because, despite increased awareness about the risks of drinking during pregnancy, a significant number of women continue to do so. Based on a large household survey, the CDC estimates that 1 in 13 women drink alcohol during pregnancy (CDC 2012). Studies interviewing women just after birth have found that between 25 and 35 percent of newborns were exposed to alcohol in utero (Gauthier et al. 2005*a*; Lester et al. 2001). Interestingly, and contrary to many traditional biases (Goldberg 1995; Hans 1999), these studies also found that older women and women of higher socioeconomic status were as or more likely to drink during pregnancy than younger, less affluent women (CDC 2012; Gauthier 2005*a*; Hutchinson et al. 2013). Because most studies of maternal alcohol use rely on self-reports, and there remains significant stigma associated with alcohol use during pregnancy, these findings likely underestimate the true extent of this problem.

Risk of Alcohol Exposure in Term Infants

Although full-term babies generally are healthier compared with babies born prematurely, there is some evidence that maternal alcohol exposure can increase the risk of neonatal infection even in term newborns. One study, for example, evaluated neonatal infections in 872 newborns with gestational age greater than or equal to 36 weeks. Infants whose mothers reported any alcohol use, excessive drinking, or smoking during pregnancy were more likely to have an infection than infants whose mothers reported that they abstained from alcohol ingestion or cigarette smoking (Gauthier et al. 2005*a*). When the researchers controlled for race and smoking, infants that were small for gestational age (SGA) and whose mothers used any alcohol had a 2.5-fold increased risk of infection. Excessive alcohol use by the mother in these SGA infants increased the risk of infection three- to fourfold. Even after controlling for low maternal income, smoking, and having a baby that was SGA, the researchers found that the newborns were three times more likely to have a neonatal infection if their mothers drank more than seven drinks per week during pregnancy (Gauthier et al. 2005*a*). This effect was most significant if the alcohol use occurred in the second trimester of pregnancy, a time when the neonatal immune system is developing. These

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findings suggest that maternal alcohol ingestion may increase the risk of potentially serious acute health problems in the postnatal period, even in full-term infants. Risks of alcohol exposure are even more significant for those babies born prematurely. We will therefore focus the remainder of the article on this uniquely vulnerable population.

Alcohol's Link to Premature Birth

Premature infants are at increased risk for a variety of significant medical complications, including respiratory, cardiac, neurological, and gastrointestinal problems as well as infection and infection-related complications. Alcohol consumed during pregnancy, researchers postulate, may exacerbate these problems. In addition, research continues to evaluate the hypothesis that drinking during pregnancy can independently increase the risk of premature birth.

The strength of the potential link between alcohol and premature birth remains under debate, because several studies have failed to demonstrate a significant relationship between alcohol and prematurity (Bailey and Sokol 2008). However, Bailey and Sokol (2008) argue that the suspected link is strengthened if they account for potential flaws in study design, particularly among women who drink heavily or binge drink during pregnancy (Bailey and Sokol 2011). In fact, the data thus far do not demonstrate a link between low-to-moderate drinking during pregnancy and the risk of premature delivery (Bailey and Sokol 2011), but multiple studies demonstrate a two- to threefold increase in the risk of premature delivery for women who drink heavily or binge drink during pregnancy (Kesmodel et al. 2000; Mullally et al. 2011; O'Leary et al. 2009; Sokol et al. 2007). Furthermore, heavy drinkers exhibited a dramatic 35-fold increased risk of delivering their babies extremely prematurely (earlier than 32 weeks) compared with women who did not drink during pregnancy (Mullally et al. 2011; Sokol et al. 2007). Therefore, some authors propose that extreme prematurity is an alcohol-related birth defect (Sokol et al. 2007).

Maternal alcohol use also has been associated with multiple risk factors that independently increase the risk of premature delivery. For example, chorioamnionitis—an inflammation of the fetal membranes due to a bacterial infection—confers a significant risk for preterm labor and premature delivery and also increases the risk of multiple adverse outcomes for premature newborns (Pappas et al. 2014). In multiple reviews, maternal alcohol use significantly increased the risk of chorioamnionitis, with risks ranging from five to more than seven times higher when compared with pregnancies without alcohol exposure (de Wit et al. 2013; Hutchinson et al. 2013). Placental abruption, a dangerous condition when the placental lining separates from the uterus, also increases the risk of premature delivery (Sokol et al. 2007). A large review of risk factors for placental abruption suggested that maternal alcohol ingestion increased the risk of abruption by more than twofold (Martinelli et al. 2012).

Although these findings suggest that maternal alcohol use is a risk factor for premature delivery, identification of alcohol-exposed term and premature newborns using traditional clinical tools is poor in both the well-baby nursery as well as newborn intensive care units (Little et al. 1990; Stoler and Holmes 1999). Given this, in order to accurately determine alcohol's adverse effects on premature newborns, it is paramount to validate biomarkers of alcohol exposure in this already at-risk population. One potential marker is a product of alcohol metabolism called fatty acid ethyl esters, which studies suggest accurately determine alcohol exposure in term newborns and in adults (Bearer et al. 1992, 2005; Best and Laposata 2003; Kulaga et al. 2006; Laposata and Lange 1986). Additional research examining ways to improve the accuracy of identifying alcohol-exposed newborns has evaluated the combination of other products of nonoxidative ethanol metabolism including phosphatidylethanol (PEth), ethyl glucuronide (EtG), and ethyl sulfate (EtS) (Bakhireva et al. 2014; Joya et al. 2012). To date, researchers have investigated these methods only in term pregnancies. They now need to test them in premature newborns exposed to alcohol. Once there is an accurate, safe, and convenient way to identify premature newborns exposed to alcohol, it will enable researchers to determine how prenatal alcohol exposure contributes to the development of common disorders

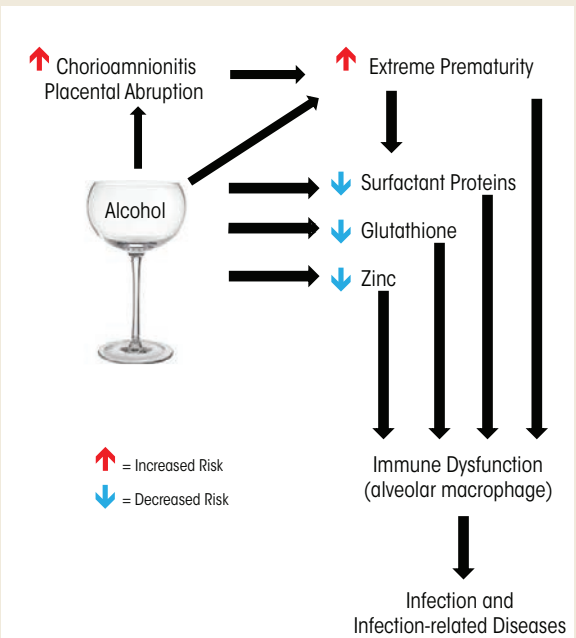

Figure Research suggests that alcohol exposure in utero affects infants' immune function through a variety of mechanisms, including indirectly by increasing the risk of premature birth and directly by influencing immune mediated defenses, particularly in the lungs.

faced by the premature population, including late-onset sepsis (infection), the lung condition bronchopulmonary dysplasia, the gastrointestinal disease necrotizing enterocolitis, and neurodevelopmental delays.

Premature Birth and the Risk of Infection

Despite a lack of biomarkers to specifically identify alcohol-exposed premature infants, research can begin to indirectly link in utero alcohol exposure to increased risk of infections and infection-related illnesses in this population. For all newborns, but particularly those born prematurely, infections play a significant role in illness and mortality (Alarcon et al. 2004; Benjamin et al. 2006; Cordero et al. 2004; Stoll et al. 2010). Even with antibiotic therapy and modern neonatal intensive care, the risk of bacterial infections remains disproportionately elevated in premature newborns and those born within minority groups (Stoll et al. 1998, 2002). Bacterial infection in the premature population increases the risk of a variety of complications including patent ductus arteriosus, in which abnormal blood flow persists between the pulmonary artery and the aorta; necrotizing enterocolitis, in which intestinal tissue becomes diseased and can die; bronchopulmonary dysplasia, a chronic and serious lung condition (Stoll et al. 2002); and neurodevelopmental delays (Adams-Chapman and Stoll 2006; Stoll et al. 2004).

Even as the premature newborn grows, it remains at increased risk for significant problems related to respiratory infections, particularly those of viral origin. Although immunization strategies such as Palivizumab, which aims to prevent serious and often life-threatening lung infections caused by respiratory syncytial virus (RSV), target premature newborns and at-risk newborns with significant lung disease, the growing premature newborn remains at an increased risk for RSV infection, particularly in the lower respiratory tract of the lung (American Academy of Pediatrics 2009; Hall et al. 2009). Furthermore, children born prematurely continue to be at increased risk for severe influenza infections, which adversely affect their long term prognosis (Izurieta et al. 2000; Louie et al. 2006).

Data directly linking in utero alcohol exposure to infections in infants and children are sparse, but some studies suggest an increased risk of neonatal bacterial infection. For example, a small study of children diagnosed with FAS found abnormal lymphocytes and increased rates of bacterial infections such as meningitis, pneumonia, and otitis (Johnson et al. 1981). In addition, hospital stays during the first year of life are approximately three times longer for infants with FAS compared with matched control infants (12.1 days vs. 3.9 days, respectively), with pneumonia being one of the main reasons for hospitalization (Kvigne et al. 2009). Drugs, including alcohol, also potentially increase the risk of maternal to fetal HIV transmission. There is a well-described association between alcohol abuse, the use of other drugs of abuse, and the acquisition and progression of HIV/AIDS among women (Wang and Ho 2011; also see the article by Bagby and colleagues).

The question remains, however, whether alcohol exacerbates the increased risk of infection already occurring in premature infants. To test this, we performed a small case-control analysis of very-low-birth-weight, premature newborns (birth weight less than 1,500 grams). We used social-work interviews to assess maternal alcohol use during pregnancy and found that premature babies exposed to alcohol in utero were 15 times more likely to show signs of early-onset bacterial sepsis than matched premature newborns without in utero alcohol exposure. This risk of early-onset bacterial sepsis with alcohol exposure remained even after we controlled for chorioamnionitis and premature prolonged rupture of membranes (Gauthier 2004). This study suggests that maternal alcohol use during pregnancy increases the risk of infection in the premature newborn, but much investigation still is necessary to fully define the influence of maternal alcohol use on neonatal infection.

Animal models of fetal ethanol exposure play an important role in furthering this research. These models help identify mechanisms underlying alcohol's detrimental effects on immune defense (Gauthier et al. 2005*b*, 2010; Lazic et al. 2007; McGill et al. 2009; Sozo et al. 2009), and they not only support these early clinical findings but also suggest that in utero exposure alters multiple arms of innate immunity in the developing fetal lung, as we discuss below.

Maternal Alcohol Ingestion and Lung Immunity

As mentioned above, viral-mediated respiratory infections can be an ongoing problem for children born prematurely. In particular, they are at increased risk for RSV and influenza. Emerging data from animal research provide insight into mechanisms underlying these findings.

Studies of animals exposed in utero to ethanol suggest that ethanol-induced immune dysfunction persists into adulthood. Specifically, adult animals exposed to ethanol in utero demonstrated impaired adaptive immunity and altered B-cell responses, resulting in increased risk and severity of influenza infection (McGill et al. 2009). Another study (Zhang 2005) demonstrated that in utero ethanol exposure alters the hypothalamic–pituitary–adrenal axis, which in turn results in hyperactivity in stress-induced immunosuppression and increased vulnerability to subsequent infectious illness.

Innate immunity in the lung is impaired in the premature newborn (Bellanti and Zeligs 1995; Hall and Sherman 1992). Growing evidence suggests that in utero ethanol exposure further disrupts multiple arms of innate immunity in the developing lung. Studies in sheep, for example, find that in utero ethanol disrupts immune function by decreasing in the fetal lung surfactant proteins (SP), which also are known as collectins, particularly SP-A and SP-D (Lazic et al. 2007; Sozo et al. 2009). In the lung, these proteins are essential mediators of the local immune response in that

they modulate the function of dendritic and T cells and facilitate the removal of pathogens by the alveolar macrophage (Sorenson et al. 2007).

The alveolar macrophage is the resident inflammatory cell that provides the initial defense against foreign and infectious particles and orchestrates the inflammatory process within the lung (Fels and Cohn 1986; Standiford et al. 1995). Alveolar macrophages reside in the lungs' alveoli and are derived from peripheral circulating blood monocytes (Fels and Cohn 1986; Prieto et al. 1994). As a consequence, anything that affects immune responses of fetal monocytes—for example, exposure to alcohol during pregnancy—may subsequently affect the alveolar macrophage population and the inflammatory environment within the newborn lung (Kramer et al. 2004, 2005).

Furthermore, substances that directly affect alveolar macrophages can therefore affect immunity in the infant lung. Studies in animals find that fetal alcohol exposure decreases the antioxidant glutathione in the fluid lining the alveolar space and within the resident alveolar macrophages (Gauthier et al. 2005*b*). Reductions in glutathione cause oxidative stress in the lung that, in turn, contributes to alveolar macrophage dysfunction and altered alveolar macrophage maturation (Brown et al. 2007; Gauthier et al. 2005*b*, 2010). Other studies in guinea pigs demonstrated that impaired alveolar macrophage function increases the already elevated risk of experimentally induced pneumonia in the newborn pup (Gauthier et al. 2009; Ping et al. 2007). Providing the pregnant guinea pig with the dietary supplement S-adenosylmethionine (SAMe) during ethanol ingestion prevented glutathione depletion in the neonatal lung, protected the neonatal alveolar macrophage from increased reactive oxygen species, improved alveolar macrophage phagocytosis, and decreased the risk of sepsis and pneumonia in the pup. In addition, giving intranasal glutathione treatments to newborn pups exposed in utero to alcohol improved macrophage phagocytosis and diminished lung infections and dissemination of experimentally induced *Group B Streptococcus* pneumonia (Gauthier et al. 2009). These findings support the idea that fetal ethanol exposure causes glutathione depletion in the lung, which in turn decreases the fetal lung's ability to clear infectious particles and increases the risk of respiratory infections.

Research in both humans and animals suggest that zinc depletion also may play a role in dampening immunity in alcohol exposed infants. Zinc is an essential cofactor in approximately 300 enzyme-dependent processes involved in immunity, growth, cell differentiation, and metabolism (Chandra 2002; Uriu-Adams et al. 2010). Studies of global disease burden for 2010 found that a primary risk factor for death in early infancy was bacterial infection linked to zinc insufficiency (Chaffee and King 2012; Lim et al. 2012; Mori et al. 2012). Indeed, zinc is essential for innate and adaptive immune responses (Knoell and Liu 2010; Maggini et al. 2007), and suboptimal concentrations of zinc result in an increased susceptibility to infection as well as exacerbation of existing infections (Prasad 2013). Newborns are at an increased risk for suboptimal zinc concentrations if their mothers have suboptimal zinc pools, and women who abuse alcohol during pregnancy tend to have suboptimal zinc pools (Keen et al. 2010; Picciano 2003). In addition, researchers have shown that decreases in zinc are a potential relative risk factor for FASD, and zinc supplements may protect against some of the adverse effects of prenatal alcohol exposure (Keen et al. 2010; Picciano 2003). Because approximately 50 percent of pregnancies are unintended (Finer and Henshaw 2006), some mothers may continue drinking during at least part of their pregnancy, resulting in significant fetal alcohol exposure and risk of suboptimal zinc concentrations in newborns. Furthermore, because the majority of zinc is transported across the placenta in the third trimester of pregnancy, newborns born prematurely, before zinc transport is complete, also are zinc deficient (Giles and Doyle 2007), which suggests that premature newborns exposed to alcohol in utero may be at an even higher risk of zinc deficiency.

A study in adult rats suggests a possible mechanism for zinc's effect on alcohol-induced alveolar macrophage dysfunction. The study found that chronic ethanol ingestion decreased the zinc levels in alveolar macrophage due to decreased expression of zinc transporters (Mehta and Guidot 2012; Mehta et al. 2011). Equally important, dietary zinc restored zinc pools in the alveolar macrophage and improved phagocytosis. Investigations in fetal ethanol models suggest that similar zinc deficiencies contribute to fetal alveolar macrophage dysfunction in the newborn.

Potential Areas for Further Research

Further research defining the mechanisms underlying alcohol-induced alterations in the immune function of the alcohol-exposed newborn is necessary. In the adult alcohol-exposed lung, alcohol-induced mitochondrial dysfunction significantly contributes to cellular dysfunction and impaired immune response of the alveolar macrophage (Liang et al. 2013, 2014).

Systemically, alcohol alters multiple arms of the immune system. Alcohol-induced increase in intestinal permeability and alterations of the gut microbiome directly contribute to alcohol-associated hepatic inflammation and the progression of liver disease (Chen and Schnabl 2014; Elamin et al. 2013; see also the article by Engen and colleagues). Alcohol-induced changes in gut permeability and the gut's interaction with the liver modulate both lung and liver inflammation in the setting of burn injury (Chen et al. 2014). Antigen presentation and T-cell dysfunction contribute to the complex immune dysfunction of the alcohol-exposed adult (Fan et al. 2011; Gurung et al. 2009). These important mechanisms have yet to be evaluated among fetuses exposed to alcohol in utero. They remain important potential areas of research particularly in the premature newborn, because morbidities such as late onset sepsis, bronchopulmonary

dysplasia, and necrotizing enterocolitis are interrelated (Stoll et al. 2010).

Conclusion

This article highlights evidence from research in humans and animals suggesting that ingesting alcohol during pregnancy can disrupt the fetal immune system and result in an increased risk of infections and disease in newborns and possibly throughout life. It also emphasizes the critical need for more research to illuminate the strength and nature of this link and the mechanisms by which alcohol may influence the developing immune system.

In particular, researchers need more specific and accurate assays for identifying which newborns have been exposed to alcohol in utero, along with methods to determine the extent and timing of such exposure. Such approaches will allow researchers to determine and more precisely measure the influence of alcohol on infections and diseases related to immune system dysfunction. In addition, continued research is needed to clarify the potential link between alcohol and premature birth, particularly extreme premature delivery.

Evidence from studies in animals has begun to provide theories about how alcohol may disrupt the developing immune system. These animal models already have begun to identify molecular mechanisms in the lung that may directly and indirectly lead to an increased risk of respiratory infections. These studies not only point to potential mechanisms of immune system disruption attributed to in utero alcohol exposure but also to possible interventions that might ameliorate the damage to the developing infant.

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Advances in Diagnosis and Treatment of Fetal Alcohol Spectrum Disorders

From Animal Models to Human Studies

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Prenatal alcohol exposure can cause a number of physical, behavioral, cognitive, and neural impairments, collectively known as fetal alcohol spectrum disorders (FASD). This article examines basic research that has been or could be translated into practical applications for the diagnosis or treatment of FASD. Diagnosing FASD continues to be a challenge, but advances are being made at both basic science and clinical levels. These include identification of biomarkers, recognition of subtle facial characteristics of exposure, and examination of the relation between face, brain, and behavior. Basic research also is pointing toward potential new interventions for FASD involving pharmacotherapies, nutritional therapies, and exercise interventions. Although researchers have assessed the majority of these treatments in animal models of FASD, a limited number of recent clinical studies exist. An assessment of this literature suggests that targeted interventions can improve some impairments resulting from developmental alcohol exposure. However, combining interventions may prove more efficacious. Ultimately, advances in basic and clinical sciences may translate to clinical care, improving both diagnosis and treatment.

Key words: Fetal alcohol spectrum disorders; prenatal alcohol exposure; fetal alcohol effects; developmental alcohol exposure; developmental disorder; diagnosis; treatment; intervention; human studies; clinical studies; animal models; literature review

Alcohol consumption during pregnancy can interfere with both embryonic and fetal development, producing a wide range of outcomes that fall under the rubric of fetal alcohol spectrum disorders (FASD). FASD is the nondiagnostic umbrella term used to refer to the full range of effects that can occur following prenatal alcohol exposure. Such exposure can produce a variety of effects, including physical birth defects, growth retardation, and facial dysmorphism, but the most profound effects are on the developing brain and accompanying cognition and behavior. The disabilities associated with prenatal alcohol are variable, influenced by numerous factors, and can have a lifelong impact. Therefore, early diagnosis and intervention are essential for improved clinical outcomes (Streissguth et al. 2004).

Animal models have played a critical role in research on FASD, including studies confirming that alcohol is indeed a teratogen and those providing insights into the mechanisms by which alcohol exerts its teratogenic effect. Researchers have used a wide variety of organisms to model the effects of prenatal alcohol exposure, which mimic both the physical and the behavioral alterations seen in human FASD (Wilson and Cudd 2011). These models allow researchers to experimentally control factors, including alcohol dose, pattern and timing of exposure, nutritional status, maternal factors, and genetics, that are known to influence and contribute to variability in clinical outcomes. Animal models also can help identify better strategies for diagnosing and treating FASD. This review will not directly compare the animal and human data because previous reviews have done this (Schneider et al. 2011). Rather, it will highlight and integrate translational research that might lead to advancements in the diagnosis and treatment of FASD. Furthermore, several psychosocial, academic, and

behavioral interventions for FASD that recently have been discussed elsewhere (Paley and O'Connor 2011) are difficult to model in animals and thus will not be reviewed here. Instead, this review focuses on recent pharmacological, nutritional, and exercise interventions that have shown promise in preclinical studies and are progressing toward translation to the clinic.

Identification and Diagnosis

To obtain an accurate estimate of FASD prevalence and provide early intervention for affected individuals, it is critical to identify infants prenatally exposed to alcohol. Identification is less problematic on the severe end of the spectrum—where fetal alcohol syndrome (FAS) lies—because it is characterized by obvious growth retardation, central nervous system (CNS) dysfunction, and a specific pattern of craniofacial anomalies (see figure 1A). However, many, if not the majority, of individuals affected by prenatal alcohol exposure do not meet criteria for FAS (Bertrand et al. 2005), yet have significant neurobehavioral impairments (Mattson et al. 2013). These cases are referred to as alcohol-related neurodevelopmental disorders (ARND) and are often difficult to identify because they lack the characteristic facial features and growth retardation seen in FAS. In fact, an ARND diagnosis requires confirmation of prenatal alcohol exposure, which often is unavailable or unreliable (see Riley et al. 2011 for a comparison of various diagnostic schemas for FAS and ARND). Finding novel ways to identify at-risk individuals for disabilities along the spectrum is critical, as is identifying effective interventions to mitigate these cognitive and behavioral effects.

The routine use of objective, validated, and highly specific markers of prenatal alcohol exposure would help improve FASD identification, which currently is hampered by a lack of good information. For example, a recent study (May et al. 2014*a*) found that only 33 percent of the mothers of children given a diagnosis of FAS provided information about their alcohol consumption. In addition, a large number of children with FASD are in adoptive situations or foster care, and there may be little knowledge of their alcohol exposure. Several indirect and direct markers of alcohol exposure (see figure 2A) exist and have been described at length elsewhere (Bakhireva and Savage 2011). Fatty acid ethyl esters, ethyl glucuronide, ethyl sulphate, and the alcohol-derived phospholipid phosphatidylethanol are among several promising metabolic biomarkers. All of these are byproducts of alcohol metabolism, and each is limited by how long after alcohol exposure they are detectable. Another newly identified marker may persist longer than these metabolic markers. As shown in a sheep model, unique circulating microRNAs (miRNA) may help identify individuals consuming alcohol and, importantly, those exposed to alcohol in utero. An initial study

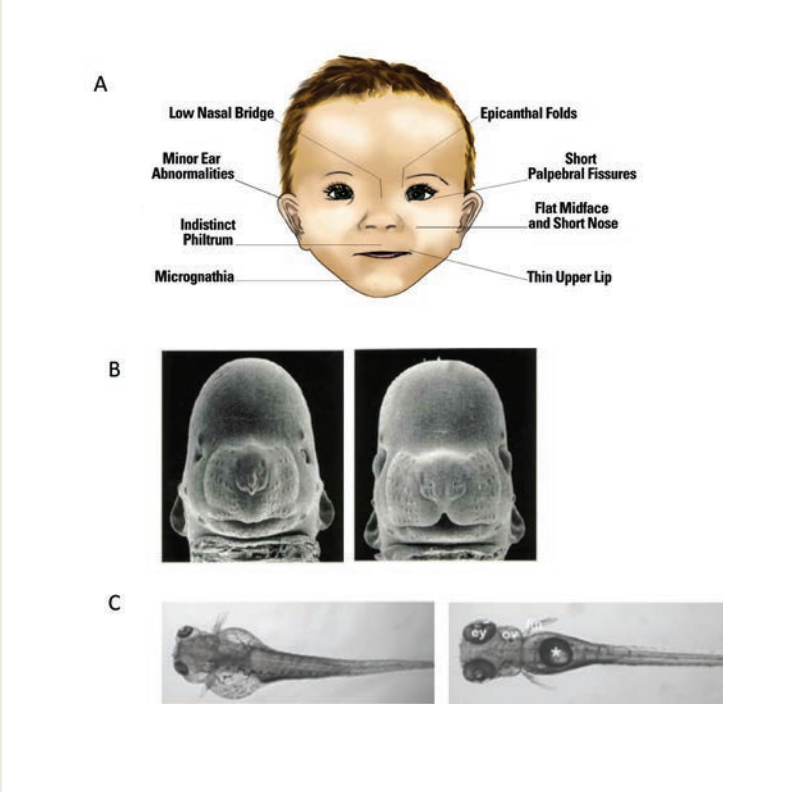

Figure 1 Craniofacial anomalies associated with alcohol exposure during development. **(A)** An illustration of a child with facial features of fetal alcohol syndrome (FAS). **(B)** Left figure shows a mouse with gestational day 7 alcohol exposure: Note small head, small eyes, and lack of a cleft under the nose compared with the control mouse on the right. **(C)** Zebrafish with embryonic alcohol exposure on the left compared with a control on the right. Again notice the small eyes, the smaller head, and the malformed body cavity and fin displacement resulting from alcohol exposure.

SOURCE: Figure 1A: Warren et al. 2011.
Photos in B are courtesy of Dr. Kathleen Sulik, University of North Carolina at Chapel Hill.
Photos in C were taken from Marrs et al. 2010.

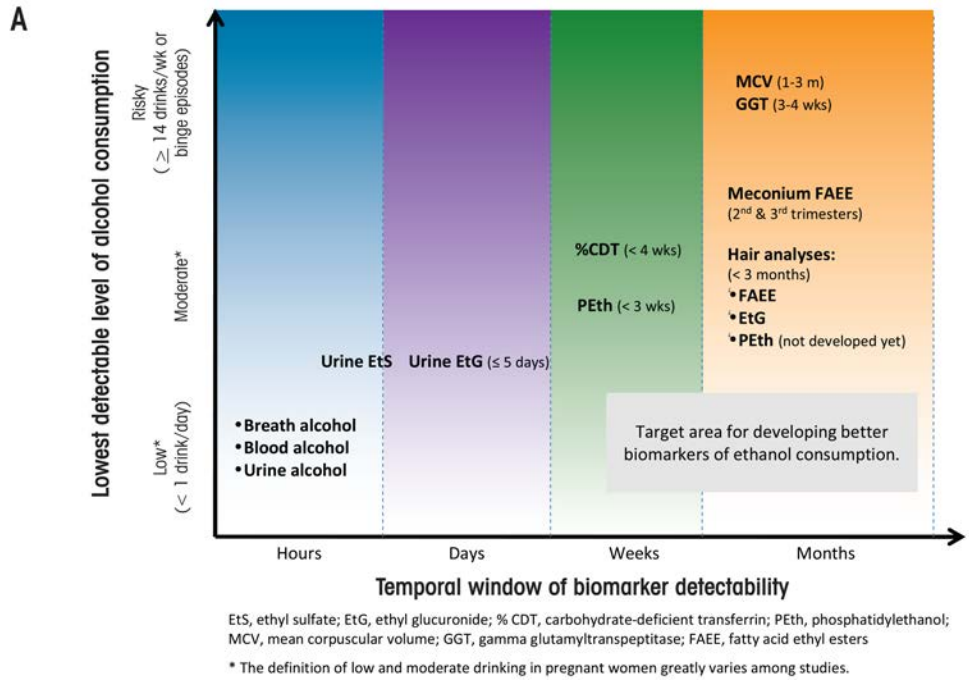

Figure 2 Indirect and direct markers of alcohol exposure. **(A)** Ideally, biomarkers could be both sensitive and specific to alcohol exposure and also indicate the timing and amount of alcohol exposure. This figure shows the period of time, or detection window, during which alcohol consumption can be detected and the lowest levels of alcohol consumption detectable by current alcohol biomarkers. For example, fatty acid ethyl esters are detectable in a variety of biological samples, such as neonatal hair and meconium, for several months after exposure. **(B)** MicroRNAs (miRNAs) may serve as potential biomarkers. Using a sheep model, Dr. Rajesh Miranda has identified several miRNAs that are modified by ethanol. As shown in this panel, miR-9 expression was significantly increased in plasma from the ethanol-exposed pregnant female compared with the control female but significantly decreased in plasma from neonatal lamb compared with controls. Alterations in miR-9 may be indicative of alcohol exposure in the mother, but also may serve as a marker of alcohol-induced injury in the neonate.

SOURCE: Figure 2(A): Bakhireva and Savage 2011. Figure 2(B): Modified from Balaraman et al. 2014.
NOTE: * = significantly different from control.

suggests that several microRNAs (miRNAs), including miR-9, -15b, -19b, and -20a, are potentially sensitive indices of alcohol exposure in both the pregnant ewe and newborn lamb (Balaraman et al. 2014) (see figure 2B). Researchers are conducting miRNA studies in humans to confirm the sheep findings. If they succeed, miRNAs may provide a new tool to identify alcohol-exposed pregnancies/infants, similar to their use as diagnostic biomarkers in a variety of other disease states (Weiland et al. 2012).

Other novel FASD diagnostic techniques include ways to identify potential at-risk individuals based upon subtle, subclinical facial features. In particular, researchers have developed a computerized method for detecting facial features using three-dimensional facial imaging and computer-based dense-surface modeling (see figure 3). Hammond and colleagues (Suttie et al. 2013) compared this approach with a standard dysmorphology exam for diagnosing FAS and found a high degree of agreement. The researchers used sophisticated mathematical techniques to characterize the facial features of heavily exposed individuals who did not have facial features that would have led to a diagnosis of FAS using traditional measures. They categorized participants as having facial features that were either "more similar to those with FAS" or "more similar to unexposed controls." Importantly, the heavily exposed children with FAS-like faces performed at a level similar to the FAS group on neurobehavioral tests, whereas those with more control-like faces exhibited behavioral profiles similar to control subjects. These data were collected on a homogenous ethnic group in South Africa and therefore need to be replicated in other populations. Still, they provide preliminary evidence that this approach may constitute a means to identify at-risk individuals based upon subtle, sub-clinical facial features.

Developing truly accurate and specific methods for identifying individuals with FASD requires an understanding of the full spectrum of alcohol-related consequences and clarification of the various factors, both protective and permissive, that influence outcome variability. Animal models have provided information on the mechanisms by which alcohol affects facial development and the factors that may make a fetus more susceptible to these facial changes (see figure 1B and C for examples of craniofacial defects in the mouse and zebrafish). In the mouse, for example, alcohol administration on gestational

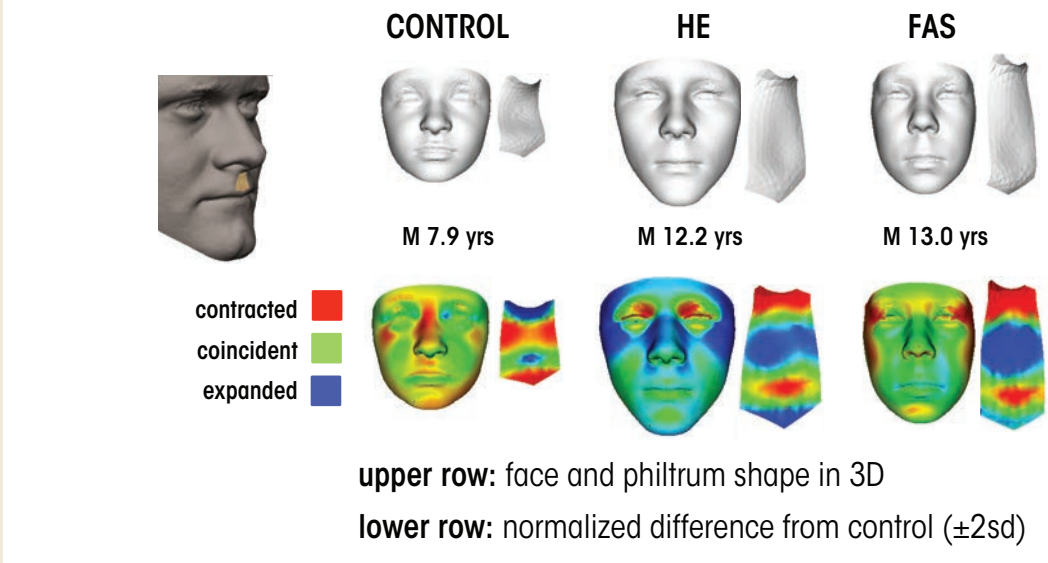

Figure 3 Three-dimensional facial imaging used to detect the effects of prenatal alcohol exposure. Each case shows face and philtrum (ridge under nose) shape as well as heat maps indicating significant regions of difference from age- and sex-matched control subjects. The control case shows an unexposed individual with some flattening across the nasal bridge, a small jaw and a strongly grooved philtrum. The heavily exposed (HE) case is an individual with known exposure without clinically recognized fetal alcohol syndrome (FAS). The overall face size is average or larger and the upper part of philtrum is smooth. The FAS case shows a reduced face size and philtrum smoothness, best revealed in the philtrum heat map; red at outer canthi (outer edge of eye) identifies narrow palpebral fissures.

day (GD) 7, equivalent to approximately week 3 postfertilization in a human pregnancy, produces a constellation of facial malformations similar to those seen in FAS. Defects include severe midfacial hypoplasia, shortening of the palpebral fissures, an elongated upper lip, and deficient philtrum (Godin et al. 2010). However, alcohol exposure delayed a day and a half to GD 8.5 produces a distinctly different pattern of malformations, with mild hypoplasia and shortening of the palpebral fissures and upper lip but a preserved philtrum (Lipinski et al. 2012) (see figure 4A and B). These data suggest that maternal alcohol consumption, even before many women are aware that they are pregnant, can cause significant and selective facial alterations in their offspring. The distinctive facial phenotype of FAS depends on the timing of exposure, and other facial characteristics resulting from alcohol exposure during different critical periods are possible.

As with facial dysmorphology, basic science models illustrate that the timing of alcohol administration also produces differing patterns of brain malformations, which again may account for the variability in outcomes. O'Leary-Moore and colleagues (2011) recently reviewed the different brain changes following a single day of alcohol exposure during early fetal development in the mouse using magnetic resonance imaging (MRI). Alcohol exposure on GD 7 was particularly damaging to medial forebrain regions, with relative sparing of mesencephalic and rhombencephalic regions (Godin et al. 2010). The morphological changes induced by alcohol exposure on GD 8 included disproportionate volume reductions in the olfactory bulbs, hippocampus, and cerebellum and relative sparring of the pituitary and septal regions (Parnell et al. 2009). GD 9 exposure produced reductions in cerebellar volume, ventricle enlargement, and shape deviations in the cerebral cortex, hippocampus, and right striatum (Parnell et al. 2013). In contrast, offspring exposed to alcohol on GD 10 displayed enlarged ventri-

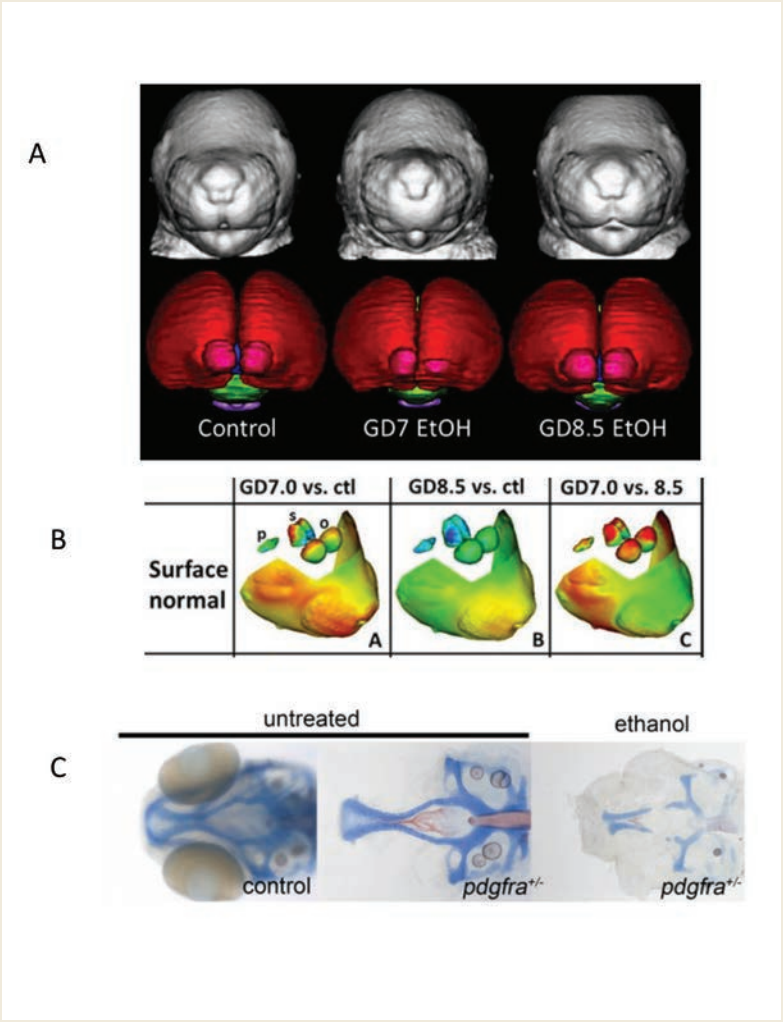

Figure 4 Magnetic resonance imaging (MRI) images showing the differential effect of different timing of exposure on face shape and brain morphology. **(A)** The left panel shows a control, whereas the two other panels show animals exposed on gestation day 7 and gestation day 8.5. The different timing produces differential effects on face and brain. **(B)** An illustration of how the shape analysis shown in figure 3 can be applied to the mouse images. The left panel shows the difference between an animal exposed on gestation day 7 versus a control. Red areas indicate a reduction in size. The middle panel shows gestation day 8 exposure versus control, note the absence of many red areas. The right panel shows the difference between the two exposure times. **(C)** Ethanol interacts synergistically with the *PDGFRA* gene. The two left most figures show an intact embryo and the dissected neurocranium of a stained *PDGFRA* heterozygote displaying normal morphology of the neurocranium. The right most panel shows how ethanol severely disrupts development of the anterior neurocranium and palate of the zebrafish. The homozygote, -/-, (not shown) is even more affected.

SOURCE: Photos in A and B are courtesy of Dr. Kathleen Sulik, University of North Carolina at Chapel Hill. Photos in C are courtesy of Dr. Johann Eberhart, University of Texas at Austin.

cles and disproportionate reductions in cortical volume (O'Leary-Moore et al. 2010). Brain-imaging studies in humans with FASD also find morphological alterations in many of these brain structures (see Moore et al. 2014 for review), which may vary depending on the specific timing of alcohol exposure. These exposure timing–dependent brain changes likely produce different behavioral outcomes, contributing to the variability in impairment seen clinically. Ultimately, understanding the relationship between alcohol exposure parameters and variability in outcome, including different behavioral phenotypes, may improve detection of individuals with FASD.

Recent studies also suggest that the interaction of alcohol with specific genes involved in brain development and the development of facial features may affect the FASD phenotype. A study in zebrafish, for example, examined the interaction of alcohol with the gene for platelet-derived growth factor receptor alpha (Pdgfra) (McCarthy et al. 2013). This gene is involved in cellular migration and proliferation and is necessary for proper migration of neural crest cells, which contribute to the formation of diverse structures, including the face. The researchers found that pdgfra interacts with alcohol to protect against severe craniofacial defects. Specifically, more than 60 percent of zebrafish heterozygous for the pdgfra gene showed cranial facial defects after alcohol exposure compared with only about 10 percent of the alcohol-treated wild-type embryos (figure 4C). A genome-wide genetic scan, using single nucleotide polymorphisms (SNPs), in humans with FASD supports these findings, showing that craniofacial phenotypes seen in FASD are linked to the *PDGFRA* gene (McCarthy et al. 2013). A more recent

Glossary

Apoptosis: A process of programmed cell death.

Brain-derived neurotrophic factor (BDNF): A protein secreted in the brain to support the survival of neurons; it plays a role in the growth, differentiation, and maintenance of these cells.

Cerebellum: An area of the brain important for coordinating motor function, as well as playing a role in simple learning and attention.

Corpus callosum: A wide bundle of fibers that connects the left and right hemispheres of the brain.

Cortex: The outer layer of the brain that is composed of folded gray matter and associated with perception, voluntary movement, and integration of information to support cognitive functions such as memory, language, and abstract thinking, among others.

cAMP response element–binding protein (CREB): A protein that binds to certain stretches of DNA and influences activation of genes.

Epigenetics: The study of factors that affect gene expression without directly changing the DNA.

Epigenome: Chemical changes to the DNA and histone proteins that affect gene expression.

Ethyl glucuronide: A byproduct of alcohol metabolism formed in the body after alcohol consumption.

Ethyl sulphate: A byproduct of alcohol metabolism formed in the body after alcohol consumption.

Fatty acid ethyl esters: The products of a reaction between ethanol and fatty acid cells.

NMDA receptors: A receptor in the brain activated by the neurotransmitter glutamate. Among its many roles, NMDA receptors help control synaptic plasticity (the ability of the brain to change and evolve), learning and memory.

Oxidative stress: When there is an imbalance between the body's production of reactive oxygen species (free radicals), and antioxidants, which defend against reactive oxygen species.

Pallidum: Refers to the globus pallidus, a subcortical brain structure involved in the regulation of voluntary movement.

Palpebral fissures: The opening between the upper and lower eyelids; length is measured as the distance between the inner to outer eye corners.

Peptide: Chains of 10 to 50 amino acids.

Philtrum: The typically vertical groove between the upper lip and nose.

Phosphatidylethanol: A metabolite of alcohol, created when phospholipase D interacts with alcohol.

Teratogen: A substance that interferes with development and causes birth defects.

Thalamus: A part of the vertebrate brain made up of two symmetrical halves deep in the middle of the brain. Among other roles, it is involved in relaying sensory and motor signals to the cerebral cortex, and regulating consciousness, sleep, and alertness.

study in zebrafish found that a gene involved in the development of the embryonic axis, *vangl2,* interacts strongly with alcohol (Swartz et al. 2014). This finding provides another potential gene target to help identify significant sources of variance in terms of susceptibility to the facial characteristics and perhaps changes in brain seen in FASD (see McCarthy and Eberhart 2014 for a recent review of genetic factors involved in FASD).

Basic research in people with FASD also is providing new methods for assessing alcohol's clinical effects. Studies have identified several relationships between facial measurements and brain structure in FASD (reviewed in Moore et al. 2014). For example, shorter palpebral fissures predict volume reductions in the bilateral ventral diencephalon, a thinner anterior corpus callosum, and a thicker right inferior frontal cortex. The smoothness of the philtrum predicts volumetric reductions in the thalamus and the left pallidum. Facial measures also predict brain maturation patterns: Children with greater facial dysmorphia displayed a linear pattern of cerebral cortex growth, at least from childhood through adolescence, rather than the developmentally appropriate inverted U-shaped trajectory. Continued research examining the relationship between face, brain, and behavioral outcomes resulting from prenatal alcohol eventually may lead to the identification of specific patterns of anomalies that can be used to better identify FASD and improve diagnosis. Moreover, patterns of outcomes may illuminate mechanisms by which alcohol disrupts developmental processes, which can inform treatment strategies. It must be cautioned, however, that the utility of these findings will largely depend on their sensitivity and specificity to alcohol.

Treatment Strategies

Although no specific treatments exist that are unique for FASD, the similarity between the cognitive and behavioral characteristics of FASD and other disorders provides a framework for treatment development. For example, estimates indicate that anywhere from around 50 percent to over 90 percent of individuals with FASD who have been clinically referred meet diagnostic criteria for attention deficit/hyperactivity disorder (ADHD) (Bhatara et al. 2006; Fryer et al. 2007). One approach would be to treat individuals with FASD with medications, such as stimulants, that have been successful in treating ADHD. However, mixed results have been found with stimulant treatment in clinical studies on FASD. For example, treatment with stimulant medications may reduce hyperactivity, with little evidence for beneficial effects on attention (e.g., Doig et al. 2008). Other studies have noted variable and unpredictable effects (O'Malley and Nanson 2002) or even poorer outcomes (Frankel et al. 2006) in FASD. Animal studies find that perinatal alcohol exposure leads to hyperactivity and that treatment with stimulants later in life increases, rather than attenuates, animals' spontaneous locomotor behaviors (Hannigan and Berman 2000). Atomoxetine (Strattera), a nonstimulant medication for ADHD, also is often used in the treatment of attention problems in FASD and a clinical trial of its effectiveness in FASD is under way.

Researchers are using their knowledge of the mechanisms underlying alcohol's toxic effect on the fetus to design preclinical models that test the efficacy of a number of pharmaceutical agents to mitigate alcohol-related impairments (Idrus and Thomas 2011). For example, prenatal alcohol exposure results in deficient activation of cyclic-AMP response element–binding protein (CREB), which can impair brain plasticity, a process of neural change important for brain development, learning, and memory. The pharmaceutical vinpocetine, a vasodilator and anti-inflammatory agent, inhibits the enzyme phosphodiesterase type 1, an action that prolongs CREB activation and thereby strengthens synaptic connections. Studies in animal models find that vinpocetine attenuates alcohol-related impairments in cortical plasticity and reduces learning and memory deficits associated with developmental alcohol exposure (Medina 2011). Clinical trials in humans with dementia have shown some promise and no serious adverse consequences, although results with other disorders, such as ischemic stroke remain inconclusive (Medina 2011). Clinical studies to evaluate this drug in humans with FASD are an important next step.

Preclinical models of FASD also have used neuroprotective peptides to mitigate neuropathologies and behavioral impairments resulting from developmental alcohol exposure. Originally, researchers administered the neuroactive peptides NAP and SAL concurrently with alcohol to pregnant rodents in an attempt to prevent alcohol-induced damage in the offspring. Subsequently, researchers have administered the peptides to adolescent rodents exposed to alcohol prenatally and found that they can reduce deficits in behavioral tasks, such as a T-maze and a Morris water maze (Incerti et al. 2010). The peptides also reversed alcohol-related changes in NMDA receptors in the hippocampus and cortex. These peptides are being developed to treat a number of neurodegenerative diseases and may prove useful in the treatment of FASD.

Nutritional Interventions

Research clearly shows that nutritional factors influence alcohol's damaging effects on the fetus. Moreover, it is possible that postnatal nutrition also might influence physical and behavioral outcomes in individuals with FASD.

Prenatal Nutritional Interventions

Some studies suggest that women who drink during pregnancy have nutritional deficits relative to control subjects. In one study, for example, May and colleagues (2014*b*) examined the

nutritional status of a group of South African mothers who gave birth to children with FASD compared with a group of mothers who gave birth to children without FASD. The mothers of children with FASD were more likely to be deficient in several vitamins, including vitamins A, B6, choline, C, D, and E; minerals, including calcium, iron, and zinc; and omega-3 fatty acids. Deficiencies in these micronutrients during pregnancy can contribute to abnormal fetal development (Nyaradi et al. 2013) and may further exacerbate the damaging effects of alcohol on the developing embryo and fetus. In animal models, maternal nutritional deficiencies (e.g., zinc or iron) during pregnancy increase the detrimental effects of prenatal ethanol on brain development and subsequent behavior in offspring. For example, the combined insults of prenatal alcohol exposure and iron deficiency resulted in increased cerebellar apoptosis (cell death), reduced myelin content, and greater impairments in cerebellar-dependent classical eyeblink conditioning compared with either insult alone (Rufer et al. 2012).

Research also finds that nutritional supplementation during pregnancy may attenuate ethanol's teratogenic effects. In one relatively small study (Avalos et al. 2011), low to moderate alcohol consumption during pregnancy resulted in a twofold increase in small-for-gestational-age infants relative to mothers who abstained. However, the offspring of women who consumed alcohol and reported taking nutritional supplements during pregnancy were no different on these measures than the offspring of abstainers (Avalos et al. 2011). The study reported similar results for preterm births. In a study of pregnant women currently being conducted in the Ukraine, researchers compared the birth outcomes of women given vitamin supplements with those not given supplements. Both groups included women who were consuming alcohol. Although the researchers still are analyzing the results, preliminary reports indicate that the women consuming alcohol and taking micronutrient supplements have a lower rate of babies with FASD than women in the nonsupplement group (Chambers et al. 2013).

Other nutritional interventions target oxidative stress. Alcohol increases oxidative stress, which in turn can initiate a cascade of events that eventually lead to widespread CNS cell loss during development (Brocardo et al. 2011). In rodent models of FASD, pregnant females given nutrients high in antioxidant properties (e.g., vitamin C, vitamin E, omega-3 fatty acids) during the time they also are given alcohol, give birth to offspring with reduced oxidative stress and cell loss, and fewer behavioral impairments (Brocardo et al. 2011; Patten et al. 2013*a*). Although antioxidant treatments in animal models are encouraging, researchers prematurely terminated a clinical trial utilizing high doses of vitamins C and E in women with alcohol-exposed pregnancies because of safety concerns (Goh et al. 2007).

Other studies are examining the role of nutritional supplements on gene transcription. Animal models of FASD demonstrate that prenatal alcohol exposure significantly affects gene transcription through epigenetic modifications (Ungerer et al. 2013). Specifically, alcohol-induced changes in DNA methylation, histone modification, and noncoding RNAs may alter the expression patterns of numerous genes important for neurodevelopment and behavior. Nutrients such as choline, betaine, folic acid, methionine, and zinc can influence these epigenetic profiles and can potentially attenuate alcohol-induced changes to the epigenome. For example, supplemental choline in rats exposed to alcohol during development alters alcohol-related changes in global DNA methylation in the hippocampus and prefrontal cortex (Otero et al. 2012) and significantly attenuates ethanol-induced hypermethylation of genes in the hypothalamus (Bekdash et al. 2013). Additionally, access to a diet supplemented with nutrients that act as methyl donors normalized changes to DNA methylation patterns in embryonic tissue following a single binge exposure to alcohol in early gestation (Downing et al. 2011). These nutrient-induced changes to the epigenome may contribute to the behavioral and cognitive improvements seen in alcohol-exposed rodents following supplementation (see below).

Additional preclinical research indicates that supplementation with beta-carotene (provitamin A), nicotinamide (the amide of vitamin B3), and zinc all may reduce alcohol's effects on fetal development, including cell loss, fetal dysmorphology, and cognitive impairments (reviewed in Idrus and Thomas 2011). These animal studies highlight the protective effects that nutrient supplementation can have on development during alcohol exposure. Improving the nutritional status of pregnant women, especially those who consume alcohol, will likely result in improved outcomes in offspring.

Postnatal Nutrient Interventions

Nutritional status also can affect cognitive development throughout childhood (Bryan et al. 2004). Recent studies have examined the nutritional intake of children with FASD. Based on their dietary habits, many children with FASD are not consuming adequate or daily-recommended amounts of omega-3 fatty acids, vitamin D, and choline (figure 5A) (Fuglestad et al 2013; Werts et al. 2014). Although these studies have some limitations—including low sample sizes, comparison with national data rather than a local control group, and relying on self-reports—they do indicate that individuals with FASD ingest inadequate levels of certain nutrients and therefore may benefit from nutrient supplementation. In rodent models, administering these micronutrients during or shortly following developmental alcohol exposure significantly mitigated ethanol-induced impairments on brain and behavior (figure 5B) (Idrus and Thomas 2011; Patten et al. 2013*b*). For example, animal

models have shown that choline can attenuate ethanol's adverse effects on both brain and behavioral development when administered postnatally, long after alcohol exposure has ceased (Ryan et al. 2008).

Clinical studies currently are underway to examine the effectiveness of choline supplementation in children with FASD. Preliminary results from a study examining choline supplementation in children with FASD aged 2.5–4.9 years suggest that supplemental choline is both feasible and tolerable, with few side effects being reported (Wozniak et al. 2013). The results on behavioral measures should be available soon. In addition to nutrient supplementation, at-risk populations may benefit from better access to food naturally high in nutrients found to improve outcomes in animal studies.

Exercise Interventions

Exercise has many beneficial effects on brain and behavior outcomes. Reports in both human and rodents indicate that exercise improves learning and memory; increases circulating proteins that support brain function, such as brain-derived neurotrophic factor (BDNF); and, in rodents, increases generation of new neurons in the adult hippocampus (Voss et al. 2013). In addition, clinical studies show beneficial cognitive effects following exercise in normal aging, Alzheimer's disease, and Parkinson's disease (reviewed in Yau et al. 2014). No published studies to date have implemented an exercise intervention to improve cognitive and behavioral outcomes in individuals with FASD, but preliminary data and preclinical results are promising, as described below.

Studies suggest that running may enhance learning and memory in rodents prenatally exposed to alcohol. Rodents will run multiple kilometers per day when they have access to a running wheel, making it ideal for an exercise intervention. Indeed, access to a running wheel significantly attenuates spatial learning and memory impairments in adult rats exposed to alcohol during development (Christie et al. 2005; Thomas et al. 2008). In addition, these improvements in cognitive function following exercise are associated with exercise-induced enhancements in BDNF and adult hippocampal neurogenesis, both of which are influenced by developmental alcohol exposure (Gil-Mohapel et al. 2010).

However, the long-term effects of short periods of exercise may be limited. For example, increases in BDNF return to normal levels within 2 weeks following exercise (Gil-Mohapel et al. 2010). That said, the benefits of exercise may be prolonged through additional environmental experiences, such as those provided by raising animals in an enriched, stimulating environment. In fact, Hamilton and colleagues (2014) have found that the combination of wheel running followed by enrichment significantly increases adult neurogenesis relative to wheel running alone in alcohol-exposed rats. Similarly, exercise plus enrichment mitigates alcohol-induced impairments on behavioral tasks, such as trace eyeblink conditioning and contextual fear conditioning. Behavioral improvement was associated with increases in adult neurogenesis

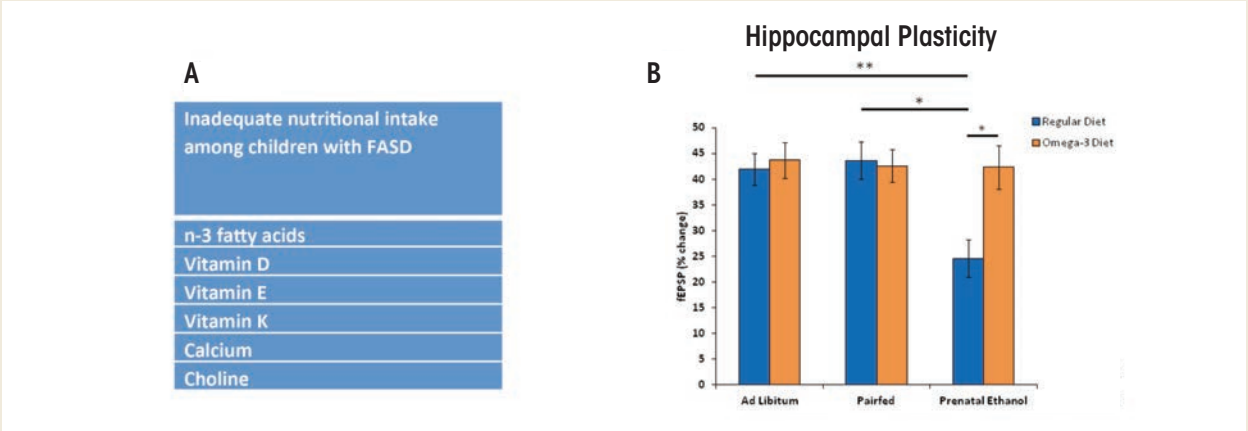

Figure 5 **(A)** Many children with fetal alcohol spectrum disorder (FASD) are not consuming adequate or recommended levels of nutrients (Fuglestad et al. 2013). **(B)** Rodent models have shown that postnatal supplementation with various nutrients, including vitamin D, choline, and omega-3 fatty acids can reduce the severity of FASD. As shown in B, prenatal alcohol exposure in a rodent model impaired hippocampal plasticity, as measured by reduced long-term potentiation (blue bars = normal diet), an effect attenuated with postnatal supplementation with omega-3 fatty acids (orange bars = omega-3 supplemented diet) (Patten, et al. 2013*b*). Such studies illustrate how preclinical and clinical studies may inform one another in the development of effective interventions for FASD.

NOTE: * = significant group differences at $p \leq 0.05$; ** = significant group differences at $p \leq 0.01$)

(Hamilton et al. 2014). In addition, specific motor training can have beneficial effects on the structure and function of the cerebellum among rodents exposed to alcohol prenatally (Klintsova et al. 2000).

In translating these preclinical findings to human studies, researchers may need to tailor their exercise interventions to accommodate some of the motor impairments evident in FASD. A recent meta-analysis of motor skills in children and adolescents with FASD reported impairments in balance, motor coordination, and ball skills (Lucas et al. 2014).

A number of clinical research programs are using these findings to develop motor training and/or exercise interventions and investigate their efficacy in individuals with FASD. None have published results yet, except in abstract form. The following are two promising examples:

- Researchers at the University of Washington are using sensorimotor training via a virtual-reality system to try to improve motor deficits. Participants stand on a moveable surface, wearing virtual-reality goggles as the program attempts to train them to use sensory information for balance (Jirkowic et al. 2014).

- Researchers at the University of the Fraser Valley are using strength-based interventions in an attempt to improve motor skills and cognitive function in FASD. In this intervention, clinicians create a physical activity and motor skills program based on an individual child's strengths, with the hope that such training may generalize to some aspects of executive functioning, attention, and visuospatial processing in children with FASD (Keiver et al. 2014).

Conclusion

FASD can be difficult to treat for a number of reasons. First, identifying individuals with prenatal alcohol exposure can be a challenge. Although the characteristics of FAS are well defined, alcohol-affected children who do not meet the criteria for FAS or for whom exposure histories are unknown are more difficult to ascertain. Children who are diagnosed earlier have improved clinical outcomes (Streissguth et al. 2004), highlighting the need for early identification. Although there are methodological and ethical concerns that must be addressed, sensitive and specific biomarkers of exposure or effect would improve identification. Continued research examining the interrelations among alcohol-induced face and brain malformations and neurocognitive outcomes using both human and animal models may yield novel means for identification and/or novel specific targets for interventions.

Overall, studies with animal models of FASD demonstrate a wide array of benefits of pharmacological, nutritional, and environmental interventions to both brain structure/function and behavior. However, relatively few clinical studies have evaluated such treatments in FASD. There are some important potential limitations to these treatments. First, many of the treatments have very specific targets and consequences, whereas the range of deficits in FASD is quite varied. For example, in animal models of FASD, nutritional supplementation with choline has a greater positive effect on hippocampal function compared with cerebellar function; in contrast, motor training may be better able to target cerebellar effects in this population. Interventions that use multiple intervention strategies (e.g., nutrition and exercise) as well as more traditional interventions (educational, speech, occupational and/or physical therapies) may mitigate a wider range of cognitive impairments when translated to clinical cases of FASD. Given the numerous successes in identifying potential interventions in preclinical research, the upcoming years should increase translation of these findings to clinical research and eventually to health care settings.

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Utilization of Magnetic Resonance Imaging in Research Involving Animal Models of Fetal Alcohol Spectrum Disorders

Xiaojie Wang, Ph.D., and Christopher D. Kroenke, Ph.D.

It is well recognized that fetal alcohol exposure can profoundly damage the developing brain. The term fetal alcohol spectrum disorder (FASD) describes the range of deficits that result from prenatal alcohol exposure. Over the past two decades, researchers have used magnetic resonance imaging (MRI) as a noninvasive technique to characterize anatomical, physiological, and metabolic changes in the human brain that are part of FASD. As using animal models can circumvent many of the complications inherent to human studies, researchers have established and explored a number of models involving a range of species. Using MRI-based modalities, the FASD animal models have demonstrated decreased brain volume and abnormal brain shape, disrupted cellular morphology differentiation, altered neurochemistry, and blood perfusion. These animal studies have facilitated characterization of the direct effects of ethanol; in many cases identifying specific sequelae related to the timing and dose of exposure. Further, as a result of the ability to perform traditional (such as histological) analyses on animal brains following neuroimaging experiments, this work leads to improvements in the accuracy of our interpretations of neuroimaging findings in human studies.

Key words: Fetal alcohol exposure; prenatal alcohol exposure; fetal alcohol spectrum disorder; fetal alcohol effects; brain; developing brain; fetal development; central nervous system; magnetic resonance imaging; neuroimaging; animal models

Neuroimaging, particularly magnetic resonance imaging (MRI), has begun to tease apart the underlying mechanisms behind alcohol's deleterious effects on the fetus and eventually may lead to earlier detection of what can be devastating child neurodevelopmental deficits. In 1968, researchers first reported an association between prenatal alcohol exposure and what can be persistent adverse cognitive, behavioral, motor, and psychosocial outcomes, leading to the first description of fetal alcohol syndrome (FAS) (Jones and Smith 1973). FAS, as described by prenatal and/or postnatal growth retardation, central nervous system (CNS) involvement, and facial dysmorphology, represents some of the most extreme effects of maternal alcohol use. However, there is a broader spectrum of symptoms, with some individuals prenatally exposed to alcohol having significant neurobehavioral deficits but not the full FAS symptomology (Mattson et al. 1997). To better represent the effect of alcohol on children prenatally exposed to alcohol, clinicians and researchers now use the term fetal alcohol spectrum disorder (FASD) (Mattson et al. 1998).

Although researchers have established a causal relationship between fetal alcohol exposure and life-long cognitive and behavioral impairment, it remains less clear how changes in the developing brain mediate these impairments. In addition, the detection of FASD remains elusive as the diagnostic criteria of FAS/FASD typically only allow for identification of affected individuals in late childhood. Noninvasive neuroimaging techniques hold potential for both identifying the underlying mechanisms behind alcohol's deleterious effects on the central nervous system (CNS) and helping detect FAS/FASD much earlier. And although studies in humans have provided some insight into these issues, studies in animals allow researchers to ask far more detailed questions.

MRI Techniques in Humans

MRI is a safe, noninvasive neuroimaging method that allows repetitive examination of human brains. It provides relatively high spatial resolution (approximately 1 × 1 × 1 mm^3 in most modalities) and a rich toolbox that enables researchers to perform anatomical, physiological, and metabolic measurements (see sidebar on "Magnetic Resonance Imaging Techniques" for detailed descriptions of the various techniques). Over the past two decades, various MRI

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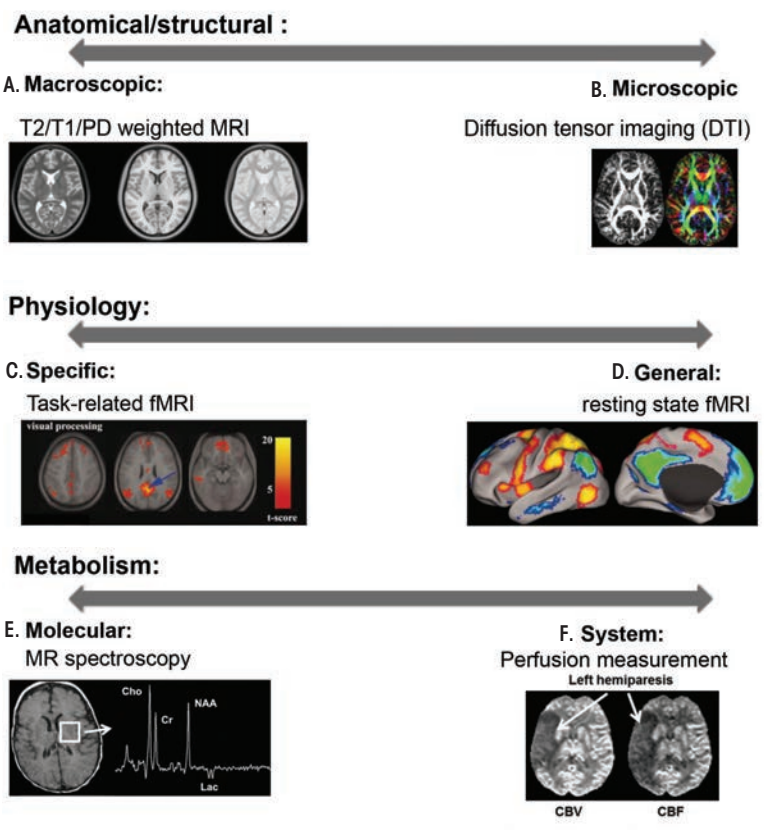
Magnetic Resonance Imaging [MRI] Techniques

To produce image contrast, conventional MRI utilizes the fact that water 1H nuclei of different tissue types have different T1 and T2 relaxation times. By varying data acquisition parameters such as time of repetition (TR) and/or time of echo (TE), contrast can be tuned to enhance differentially anatomical structures such as gray matter, white matter, and cerebrospinal fluid (CSF). As a result, imaging can segment specific anatomical structures such as basil ganglia, cerebellum, corpus callosum, and hippocampus to facilitate quantitative volume and shape analyses (for the definition of these and other terms, see Glossary).



SOURCES: C. Greicius et al. 2003; D. Fox et al. 2005; E. Gujar et al. 2005; F. Calamante et al. 1999

Diffusion Tensor Imaging (DTI)

Water diffuses through biological tissues based on thermally driven Brownian motion and is impeded by myriad structures. During the typical diffusion time in a diffusion magnetic resonance (MR) scan (10 to 100 ms), the behavior of water diffusion within the central nervous system (CNS) can vary dramatically depending on the tissue subtype. In cerebrospinal fluid, water experiences free and isotropic diffusion, which means it moves equally in all directions. In mature white matter and some gray matter regions (e.g. hippocampus, cerebellum, and cerebral cortex), interactions with biological membranes significantly reduces water diffusion perpendicular to dominant cellular processes (axons, dendrites, and glial processes). Thus, the diffusion is direction dependent, or what is known as anisotropic. In gray matter regions that lack highly oriented cellular structures, water molecules experience boundaries in a more random fashion and this situation often is referred to as restricted isotropic diffusion.

For each imaging voxel, DTI measurements can derive multiple parameters. One commonly used parameter is fractional anisotropy (FA), which characterizes the degree of anisotropy of a diffusion process. FA measurements range between 0, which represents isotropic diffusion as in free water or cerebrospinal fluid (CSF), and 1, which indicates that diffusion is completely restricted along one or more directions. Therefore, high FA reflects coherent and highly orientated fiber tracts and decreased FA often indicates myelin and axon injury, and/or any disruption of fiber tracts. Mean diffusivity (MD) is a scalar measure of the total diffusion within a voxel and reflects the mobility of water molecules. MD is generally high in CSF, and lower in normal gray and white matter. Compared with anatomical MRI, DTI-derived metrics are more sensitive to the changes on a cellular level (Mori and Zhang 2006).

Functional MRI (fMRI)

fMRI is a technique that measures brain physiological activity. It does so based on the coupling of blood flow and neuron activity and the difference in water 1H spin relaxation between environments of deoxyhemoglobin and oxyhemoglobin.

Magnetic Resonance Imaging [MRI] Techniques *continued*

In typical task-based fMRI experiments (Logothetis 2008), a subject alternates between a specific task-responding state and a control state. In brain areas where a task activates neurons, blood flow is altered such that more oxyhemoglobin is present compared to deoxyhemoglobin. This results in a transient task-dependent increase in magnetic resonance (MR) signal intensity within the brain regions that the task activates and this phenomenon is termed blood oxygen level dependent (BOLD) MR signal.

In resting-state fMRI experiments (Fox et al. 2005), no stimulus is presented to the subject, and temporally correlated MR intensity fluctuations are used to infer disparate brain areas that are functionally related to each other.

MR Spectroscopy (MRS)

MR spectroscopy provides a quantitative and specific measure of brain chemistry. While conventional MRI primarily detects water 1H nuclei, MRS detects proton signals from other molecules such as amino acids (e.g. glutamate), lipids, lactate, N-acetylaspartate (NAA), choline, creatine, and, when present, ethanol. Alternatively, MRS can also detect other MR active nuclei (e.g. 31P, 23Na, 19F, etc). Researchers can use MRI and MRS in combination: MRI to identify an anatomical location and localized MRS to detect the concentration of specific metabolites within the region of interest. Among the MRS studies of human and animal models of FASD, researchers most frequently examine NAA, choline-containing compounds (Cho), and creatine/phosphocreatine (Cr) signals. The NAA signal includes contributions from primarily NAA, and to a lesser extent from N-acetylaspartylglutamate (NAAG). NAA is considered to be a marker that reflects neuronal/axonal health, viability and density. Cho signals consist of multiple choline derivatives which are precursors or degradation products of the membrane phospholipids. Thus, the Cho signal is seen as a marker for cell membrane integrity and myelination. The Cr signal, constituted of both creatine and phosphocreatine, is thought to reflect energy phosphate metabolism. As the absolute measurements of signal intensities of these metabolites are subject to source errors including CSF contamination, the Cr peak, which is relatively constant between individuals and most brain areas, is often used as internal reference. Thus in this review, we only discuss the NAA/Cr and Cho/Cr ratios (Graaf 2002).

MR Perfusion Measurements

Cerebral blood flow (CBF), is the blood supply to the brain at any given time and is tightly regulated to meet the brain's metabolic demands. In an adult, CBF is typically 750 ml/min. This equates to an average perfusion of 50–54 ml of blood per 100 g of brain tissue per minute. A number of MR modalities can be used to measure blood perfusion within the brain, such as dynamic susceptibility contrast (DSC) MRI, dynamic contrast enhanced (DCE) MRI, and arterial spin labeling (ASL).

DSC-MRI involves the injection of a bolus paramagnetic contrast agent. Then a fast imaging sequence is used to acquire a series of T2*-weighted images during the contrast agent's first passage through the tissue. The passage of the contrast agent leads to MR signal intensity drop due to the magnetic susceptibility effect. The signal intensity-time curve measured by the series of T2*-weighted images can be mathematically converted to a contrast agent concentration-time curve. The concentration-time curve is then integrated to give an index that is proportional to the relative cerebral blood volume (rCBV) of a given imaging voxel. Additionally, if such measurement is done within or near a major artery, the arterial input function can then be derived and in turn, relative cerebral blood flow can also be calculated (Calamante 1999).

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techniques have uncovered brain abnormalities that are associated with cognitive/behavioral deficits in FASD-affected individuals (Riley et al. 1995, 2004).

Anatomical Differences

Traditional MRI studies show anatomical differences between the brains of children and adolescents with FASD and those not exposed to alcohol in utero, including the following:

- Significant reductions in overall brain volumes in children and adolescents with FASD (Archibald et al. 2001; Johnson et al. 1996; Lebel et al. 2008; Sowell et al. 2002; Willoughby et al. 2008);
- Reduced volumes in specific regions, including the caudate nucleus (Archibald et al. 2001; Cortese et al. 2006), hippocampus (Willoughby et al. 2008), and cerebellar vermis (Autti-Ramo et al. 2002); and
- Corpus callosum malformations in FASD individuals (Autti-Ramo et al. 2002; Bookstein et al. 2002; Johnson et al. 1996).

Study results are mixed regarding the effect of maternal ethanol exposure on fetal cerebral cortical thickness: Sowell and colleagues (2002, 2008) observed greater cortical thickness in parietal and posterior temporal regions, whereas Zhou and colleagues (2011) reported thinner cortical gray matter in a number of brain regions in an FASD group. Meanwhile, results from studies using diffusion tensor imaging (DTI), which allows researchers to assess tissue abnormalities on a microstructural level, even in the absence of gross dysmorphology, suggest that individuals exposed to alcohol in utero have less organized white matter fiber tracts (Wozniak and Muetzel 2011). Specifically, DTI showed significantly decreased fractional anisotropy and/or increased mean diffusivity (MD) in the corpus callosum (Ma et al. 2005) and other white matter regions (Fryer et al. 2009; Lebel et al. 2008; Sowell et al. 2008) of alcohol-exposed individual compared with those who were not exposed.

Neurochemical Changes

Another technique, magnetic resonance spectroscopy (MRS) (see sidebar on "Magnetic Resonance Imaging Techniques"), offers a unique way to detect neurochemical changes by monitoring the concentration of neurometabolites, including choline- containing compounds (Cho), which are markers of cell membrane stability and myelination; N-acetylaspartate (NAA), a marker of neuronal/axonal viability and or density; and creatine/phosphocreatine (Cr), a marker of metabolic activity (Moffett et al. 2007). One MRS study (Fagerlund et al. 2006) comparing people with FAS with normal control subjects reported lower NAA levels in various brain regions among the FAS subjects, whereas another study (Cortese et al. 2006) found higher NAA levels in the caudate nucleus.

Brain Activation Patterns

Functional MRI allows researchers to detect differences in brain activation patterns between FASD individuals and control subjects during various tasks involving spatial, verbal, and visual working memory (Astley et al. 2009; Malisza et al. 2005; O'Hare et al. 2009), verbal learning (Sowell et al. 2007), and inhibitory control (Fryer et al. 2007). These altered brain activation patterns might underlie the poor executive functioning-based skills observed in FASD individuals (Astley et al. 2009; Fryer et al. 2007; Malisza et al. 2005; O'Hare et al. 2009; Sowell et al. 2007).

Drawbacks of Human Studies

Although neuroimaging and neuropathological investigations of the brains of FASD-affected individuals have elucidated specific abnormalities in brain structure, metabolism, and function underlying cognitive and behavioral impairments, studies of human subjects have a number of limitations. These include (1) the paucity of autopsy reports from children with FASD hampers interpretation of in vivo human neuroimaging data; (2) the related inability to evaluate and validate correlative structural and functional damage; (3) the difficulty in controlling, or even determining, variables such as dosing, timing, and consumption pattern of maternal drinking; and (4) the difficulty in eliminating confounds in human studies including environment, other maternal substance abuse, stress, and malnutrition. Animal models of FAS/FASD circumvent many of these inherent complications.

MRI in Animals

Animal models allow researchers to control maternal and environmental variables such as genetic background, nutritional status, dosage, and timing pattern of ethanol insult, which frequently enables experiments to focus on the mechanisms of ethanol's teratogenic action. As early as 1977, studies of mouse and rat FASD models confirmed the causal relationship between prenatal alcohol exposure and FASD, which had been speculated in clinical observations (Abel and Dintcheff 1978; Chernoff 1977). Shortly after, Sulik and colleagues (1981) demonstrated that treating pregnant mice with alcohol at gestation day (GD) 7 (equivalent to human gestation week [GW] 3) resulted in facial dysmorphology in their offspring, a finding consistent with FASD-affected human infants. Since then, researchers have established a number of animal models in a range of species to study the mechanism of alcohol's teratogenic effects, to test the efficacy of protective interventions, and to improve the sensitivity and specificity of neuroimaging techniques for identifying FASD (see figure 1). Each model provides certain advantages and disadvantages as described below.

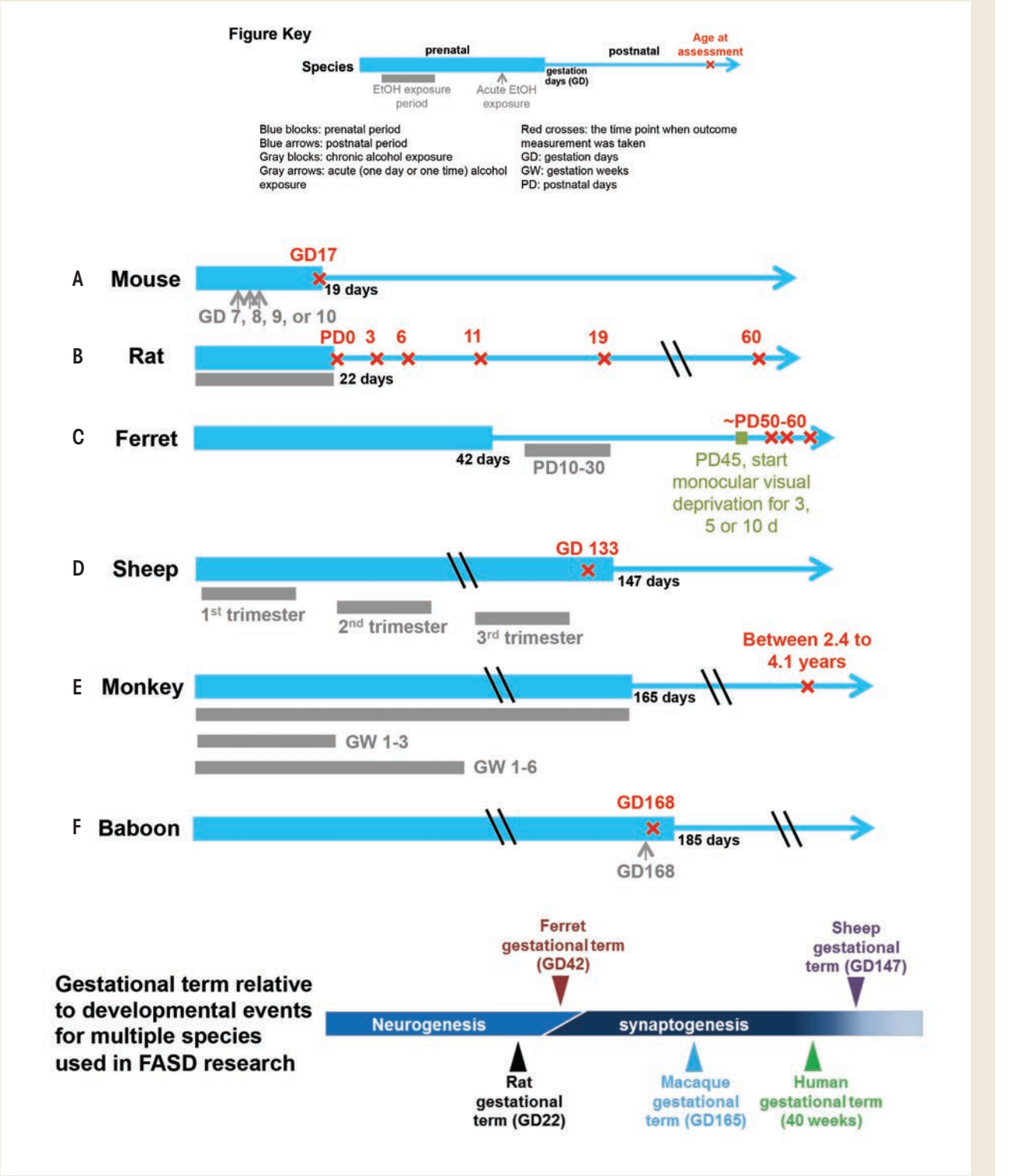

Figure 1 Timing schemes of popular animal models for FASD research.

Nonmammalian Animals

Nonmammalian animal models, including zebra fish, fruit fly, and frog, offer unique advantages by providing flexible and well-characterized experimental systems. However, more phylogenetically advanced vertebrate species are necessary in studies that require a complex CNS and long developmental periods.

Rodents

Among mammalian species with a more complex CNS, rodents are a very important model system. They have a short reproductive cycle, their genetic background is readily controlled, and their small size makes them suitable for smallbore animal MRI systems.

Ferrets

Ferrets also have a fairly short pregnancy period relative to CNS developmental milestones (see figure 1C) and, as a result, are advantageous for investigating early neurodevelopmental disruption without the complication of needing to induce premature delivery, or perform in utero manipulations.

Sheep

Sheep have a long gestational term relative to CNS development, more resembling human gestation, which allows investigators to explore various drinking patterns and exposure times more similar to those seen in humans.

Nonhuman Primates

The CNS develops very similarly in nonhuman primates and humans. In addition to their large and highly folded brains, nonhuman primates also exhibit more complex social relationships and cognitive functions. Thus, they can serve as a bridge between studies in other animal models and humans (Miranda-Dominguez et al. 2014). In a number of cases, studies conducted on FASD animal models have productively used MRI as a noninvasive neuroimaging modality. The primary outcomes from studies in all of these models, which are reviewed below, are summarized in the table.

Findings From Animal Models

Rodents

In a series of mouse FASD studies, researchers used ex vivo MRI to examine the effect of acute ethanol insult on GD 7, 8, 9, and 10, a time range that corresponds to human GWs 3 to 4 (Godin et al. 2010; O'Leary-Moore et al. 2010; Parnell et al. 2009, 2013). To characterize ethanol-induced structural brain abnormalities, they analyzed high-resolution MR images of each fetus dissected on GD 17 (see figure 2). They measured key growth metrics such as brain width, mid-sagittal brain length, and third ventricle width in a single image plane (see figure 2A). They also segmented and then reconstructed regional brain structures (e.g. cerebral cortex, ventricles, cerebellum, etc.) to quantify their volume and morphology in three dimension (see figure 2B and C). In the fetuses exposed to ethanol in utero, the researchers found notable volume reductions across various brain regions, which were accompanied by increased ventricular sizes. They also observed regional brain morphology changes including holoprosencephaly, or the absence of midline cerebral structures, and widened space between cerebral hemispheres (see figure 3B and C). These results demonstrate that an acute maternal alcohol insult on GD 7 to 10 leads to a spectrum of forebrain deficiencies in mouse fetuses. Importantly, some animals that exhibited CNS malformations did not have facial dysmorphology. This series of studies employing an acute, high-dose maternal ethanol treatment paradigm helped titrate sensitive periods for a variety of malformations and extended our knowledge of the dependency of ethanol teratogenesis on the timing of exposure during gestation.

A more recent study conducted by the same research group examined brain dysmorphology resulting from maternal dietary ethanol intake at a much lower dose than the previous study and occurring during the time period equivalent to the first trimester in humans (Parnell et al. 2014). Using the same MRI-based volumetric measurements, the researchers observed reduced cerebellum and enlarged septal region in a GD 7 to 11 ethanol-exposure group. In a GD 12 to 16 ethanol-exposure group, the researchers detected size reductions in right hippocampus and increased pituitary gland volume. Overall, the number of brain regions significantly affected and the severity of the effect were less than those following acute, high-dose exposures. The application of high-resolution MRI here has facilitated the systematic and comprehensive examination of the brain abnormalities caused by prenatal ethanol exposure. The employment of a mouse FASD model in these studies allowed the control of variables, especially ethanol exposure patterns, which, in turn, aided in confirming that the type and severity of ethanol-induced birth defects largely depend on the treatment pattern and dosage along with the developmental stage at the time of ethanol exposure (Godin et al. 2010; O'Leary-Moore et al. 2010; Parnell et al. 2009, 2013, 2014).

A study in rats using ex vivo high-resolution MRS examined regional neurochemistry in frontal cortex, striatum, hippocampus, and cerebellum in postnatal day [PD] 16 animals exposed to ethanol as neonates (PD 4 to 9) (O'Leary-Moore et al. 2008). The technique allowed them to measure the relative concentrations of certain brain metabolites, comparing the brains of ethanol exposed rats with those of control rats. They found changes in several metabolites in various brain regions:

- The NAA/Cr ratio was reduced in the cerebellum, which likely reflects delayed development, cell loss, or both in these regions. This finding supports those of a human FASD study (Fagerlund et al. 2006), reporting a decrease in NAA/Cr in cerebellum along with other brain regions. That said, another human FASD study (Cortese et al. 2006) reported an increased NAA/Cr ratio in the caudate nucleus in FASD individuals. The researchers suggested that this increase might be indicative of a "lack of normal programmed cell death, dendritic pruning/myelination during development" (p. 597).
- The Cho/Cr ratio was significantly lowered in hippocampus and elevated in striatum in ethanol-exposed rats

Table Findings of Magnetic Resonance (MR)-Based Fetal Alcohol Spectrum Disorder (FASD) Animal Studies.

MR Modalities	MR Studies	Ethanol Exposure	Age at Assessment	Findings
Anatomical MRI	Astley et al. 1995 Monkey	Weekly, gestation week (GW) 1 to 3, or 1 to 6, or 1 to 24, 2 g/kg, intragastric gavage	2 to 4 years	No gross morphological abnormalities. No gross difference in size of cerebral hemispheres, corpus callosum, brain stem, or cerebellum.
	Godin et al. 2010 Mouse	Gestation day (GD) 7, 2.9 g/kg, intraperitoneal (i.p.) injection	GD 17	Holoprosencephacy (the forebrain fails to develop), cerebral cortical heterotopia (where clumps of gray matter develop in the wrong places), failure of the pituitary gland to develop (pituitary agenesis), dilation of the third ventricle.
	Parnell et al. 2009 Mouse	GD 8, 2.9 g/kg, i.p. injection	GD 17	Reduction of total brain volume. Comparison of individual regions revealed difference in all except the pituitary and septum.
	Parnell et al. 2013 Mouse	GD 9, 2.9 g/kg, i.p. injection	GD 17	Increase in septal region width, reduction in cerebellar volume, ventricular dilation, malformation of cerebral cortex, hippocampus and right striatum.
	O'Leary-Moore et al. 2010 Mouse	GD 10, 2.9 g/kg, i.p. injection	GD 17	Ventricular dilation, reduction in total brain volume as well as each of the assessed brain structures.
	Parnell et al. 2014	GD 7 to11, 4.8 percent EtOH-containing diet (vol/vol)	GD 17	Decrease in cerebellar volume, increase in septal volume.
	Mouse	GD 12 to 16, 4.8 percent EtOH-containing diet (vol/vol)	GD 17	Reduction of right hippocampal volume, increase in pituitary volume.
	Leigland et al. 2013*a* Rat	Daily, GD 1 to 20, 4.5 g/kg, intragastric gavage	PD 0, 3, 6, 11, 19, 60	Reduction of brain and isocortical volumes, reduction of isocortical surface area and thickness.
Diffusion Tensor Imaging (DTI)	Leigland et al. 2013*b* Rat	Daily, GD 1 to 20, 4.5 g/kg, intragastric gavage	PD 0, 3, 6	Higher fraction anisotropy (FA) in cerebral cortex.
Magnetic Resonance Spectroscopy (MRS)	Astley et al. 1995 Monkey	Weekly, GW 1 to 3, or 1 to 6, or 1 to 24, 2 g/kg, intragastric gavage	2 to 4 years	Increased Cho/Cr with increased duration of EtOH intake.
	O'Leary-Moore et al. 2008 Rat	Daily, postnatal day (PD) 4 to 9, 5 g/kg intragastric gavage	PD 16	Increased NAA/Cr in cerebellum and striatum, Cho/Cr ratio was increased in striatum but decreased in hippocampus.
Perfusion MRI	Kochunov et al. 2010 Baboon	GW 24, 3 g/kg, intragastric gavage	Immediately following ethanol exposure	Increased permeability of placental membrane, increased cerebral blood flow in fetal brain.

compared with controls. The researchers concluded that these changes in Cho level were "consistent with dysfunctional membrane turnover in the young perinatal ethanol-exposed brain" (p. 1704).

- The concentration of the amino acid taurine was reduced in hippocampus and striatum. Taurine deficits can cause growth retardation and impaired CNS function (Aerts and Van Assche 2002).
- Glutamate, an excitatory neurotransmitter, was reduced in cerebellum only from prenatal ethanol-exposed female rats, indicating disrupted glutamatergic function (O'Leary-Moore et al. 2008).
- A trend of decreased γ-aminobutyric acid (GABA) (without statistical significance) also was observed in the striatum and cerebellum in the rats with neonatal ethanol exposure.

The ability to study this broad range of MRS signals in animal models may hold potential for the development of additional biomarkers for FASD diagnosis and treatment evaluation.

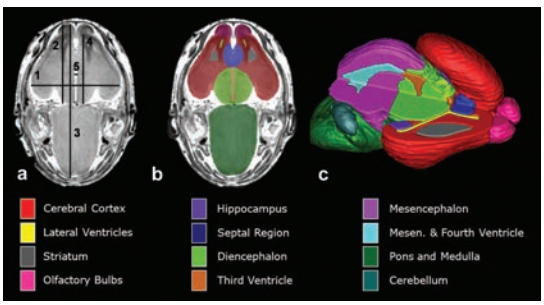

Figure 2 High-resolution magnetic resonance (MR) images of mouse fetuses at gestational day (GD) 17 allow for linear measurements, regional segmentation, and three-dimensional reconstruction. **(A)** A horizontal image with lines depicting sites of linear measurement as follows: brain width (biparietal distance), line 1; bulbothalamic distance, line 2; mid-sagittal brain length, line 3; frontothalamic distance, line 4; third ventricle width, line 5. (Cerebellar width [transverse cerebellar distance, not included] was measured at its greatest dimension.) Manual segmentation, as depicted by the color-coded regions in **(B)** allowed for subsequent three-dimensional reconstruction **(C)** and analyses of selected brain regions. **(C)** The upper right quadrant of the brain has been removed to allow for visualization of the interior structures. Color codes for the segmented brain regions shown are at the bottom of the figure.

NOTE: Figure adapted from (Godin et al. 2010).

In another rat study (Leigland et al. 2013*a*), researchers used ex vivo MRI to examine the cerebral cortex of rat pups born to dams treated with ethanol throughout gestation, comparing them with pups whose moms either received maltose/dextrin instead of ethanol or no treatment. They performed cross-sectional measurements on the pups on PD 0, 3, 6, 11, 19, and 60 (see figure 1B). The ethanol-exposed pups had reductions in volume, thickness, and surface area of the cerebral cortex on PD 0, compared with control and M/D-treated groups, and the difference persisted into adulthood (PD 60). To examine whether prenatal ethanol exposure differently affected particular areas of the cerebral cortex, the researchers analyzed differences in regional patterns of cortical thickness. They saw a significant difference in the parietal and frontal-parietal region of the cortex or somatosensory and motor locations (see figure 4). This finding agrees with a human study observing smaller cortical thickness in FASD (Zhou et al. 2011) but is at odds with reports by Sowell and colleagues (2002, 2008, 2001), in which greater cortical thickness was reported in FASD-affected individuals. The discrepancy between the two human studies might be explained by differences in the image processing procedures used. Leigland and colleagues (2013*a*), in the rat study, and Zhou and colleagues (2011), in the human study, recorded absolute cerebral cortical thickness/volume; Sowell and colleagues (2001) normalized individual gray matter volume

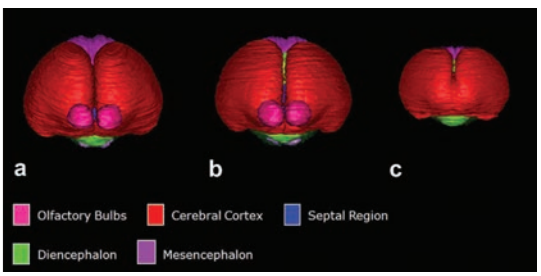

Figure 3 Reconstructed brains of a control fetal mouse at gestational age 17 **(A)** along with the brains of ethanol-exposed fetuses having mid-facial abnormality **(B** and **C)**. Segmented magnetic resonance microscopy scans of control **(A)** and ethanol-exposed **(B** and **C)** fetuses were reconstructed to yield whole brain (frontal view). Although the affected fetus in **(B)** had a normal-appearing face (figure not shown here), a slight widening of the space between the cerebral hemispheres (as evidenced by visibility of the septal region and diencephalon) can be seen as compared with control **(A)**. Missing olfactory bulb and rostral union of the cerebral hemispheres can be seen in fetus **(C)**.

NOTE: Figure adapted from (Godin et al. 2010).

to total brain volume before statistical analyses in their study. If fetal ethanol exposure disproportionately affects shrinkage of different brain structures, it is possible that differences in the direction of effect on cerebral cortical thickness could result from the different data processing strategies.

Although a majority of neuroimaging research on early cerebral cortical development has focused on gross volume change and dysmorphology, one study used ex vivo DTI on rats to characterize prenatal ethanol exposure's effect on cortical neuron morphological differentiation (Leigland et al. 2013*b*). Rats exposed to daily ethanol throughout gestation exhibited a higher diffusion fractional anisotropy (FA) in their cerebral cortex compared with age-matched M/D controls at ages PD 0, PD 3, and PD 6, indicating a higher preference for water to diffuse radially rather than parallel to the pial surface (figure 5) (see sidebar "Magnetic Resonance Imaging Techniques" for explanation of the technique). The researchers validated this finding with quantitative histological analyses of the same brains. They found that higher FA reflected a more simple and coherent cortical cellular structure, which has previously been shown with traditional invasive anatomical measurement methods (Cui et al. 2010; Davies and Smith 1981; Fabregues et al. 1985; Hammer and Scheibel 1981) to result from ethanol-induced disruption in neuronal differentiation. The framework proposed in this study in which cellular-level microstructure can be inferred by DTI-derived FA provides a novel strategy for characterizing the effects of ethanol exposure on cerebral cortical gray matter.

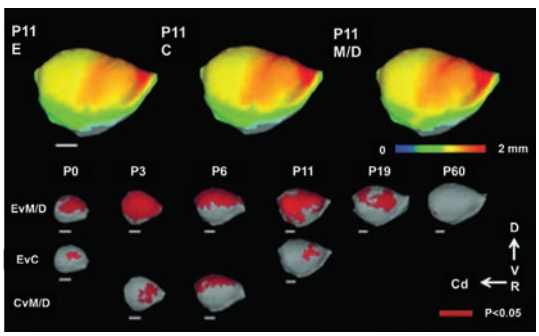

Figure 4 Regional pattern of cerebral cortical thickness differences result from threshold-free cluster enhancement (TFCE) analysis. On the top row, mean cortical thickness at postnatal day (PD) 11 for each group in the rat (n = 4 to 6/age/group) are projected onto target cortical surfaces. TFCE results are pictured in dark red in the last three rows representing regions in which mean cortical thickness between groups is significantly different ($P < 0.05$). Specific regional differences, centered on primary sensory areas were found among ethanol **(E)** and maltose/dextrin (M/D) groups at all ages. Regions of significant difference also were found in comparisons between E and control **(C)** groups at PD 0 and PD 11 and between control **(C)** and M/D groups at P 3 and P 6. Scale bars (in white) represent 2 mm. D, dorsal; V, ventral; Cd, caudal; R, rostral.

NOTE: Figure adapted from Leigland et al. 2013*a*.

Sheep and Ferret

Although, to our knowledge, no MRI studies have been published on fetal alcohol exposed sheep or ferrets, we review here some results using these species for FASD research. Gestational term lengths in these species, relative to other developmental events, represent extremes, and these properties have been exploited to address specific scientific questions. Similar to humans, sheep have a long gestation time and all three trimester equivalents occur in utero. Studies have found that binge ethanol exposure in all three trimesters leads to deficits in fetal cerebellar Purkinje cells (Ramadoss et al. 2007*a,b*) (see figure 1D). Another study using a sheep FASD model reported that second-trimester alcohol exposure has an adverse effect on fetal cerebral blood flow (Mayock et al. 2007). In contrast to sheep, ferrets have a short gestation time relative to CNS development, and its third-trimester equivalent of human gestation occurs postnatally. During this time, exposure to ethanol can disrupt neuronal differentiation, synaptogenesis, circuit formation, and remodeling of neuronal connections. Medina and colleagues (2003) have used a ferret monocular deprivation model, a well-characterized model of neuronal plasticity in the neocortex, to find that a 3-week alcohol exposure starting PD 10 impairs ocular dominance plasticity at a later age (see figure 1C), indicating ethanol insult during this time could have a profound effect on development and plasticity of neural circuits in the neocortex.

Nonhuman Primates

As early as 1995, Astley and colleagues used MRI and MRS to study brain structural and biochemical changes in a macaque monkey model of FASD (Astley et al. 1995). In this study, they explored three ethanol exposure patterns: once per week throughout the entire gestation period, once per week through GW 1 to 3, and once per week through GW 1 to 6 (see figure 1E). The researchers conducted MRI and MRS on the offspring of these treated monkeys between ages 2.4 and 4.1 years. Radiologists blinded to the monkeys' alcohol exposure inspected the MRI images and found no difference in morphology or size of cerebral hemispheres, corpus callosum, brain stem, or cerebellum. However, MRS from the thalamus, parts of the internal capsule, and basal ganglia detected a significant increase in Cho/Cr ratio with increasing duration of in utero ethanol exposure. Importantly, the study also found the Cho/Cr ratio to be associated with increased cognitive impairment as assessed by the Infant Development Impairment Score. Further analyses of NAA/Cr and NAA/Cho ratios suggested that the Cho component

changes with increasing ethanol exposure time. The researchers speculated that higher choline content might be associated with membrane breakdown.

A more recent study used dynamic susceptibility contrast (DSC)-MRI (see sidebar "Magnetic Resonance Imaging Techniques") to probe the effect of acute ethanol intake on pregnant baboons and their fetuses. Specifically, the study examined the effect of ethanol on the inner layer of the uterine wall, known as the myometrium, and on fetal brain perfusion, which is a measure of cerebral blood flow (CBF) (Kochunov et al. 2010). The researchers measured brain perfusion before (baseline) and immediately following the administration of an ethanol dose equivalent to human binge drinking (see figure 1F). In the fetal brain, the peak contrast uptake concentrations and contrast uptake and washout rates were significantly increased after ethanol treatment, suggesting that the ethanol increases CBF. The researchers hypothesized that ethanol's vasoactive properties are responsible for this CBF increase. This study also suggested that ethanol increased the permeability of placental membranes to the contrast agent, which is used to improve visibility of tissues during imaging. Specifically, the researchers found that more agent entered the fetal cerebral circulation, indicated by greater MR signal reduction in the fetal brain acutely following ethanol exposure. This is the first study to investigate ethanol's effect on fetal CBF and placenta permeability using in utero DSC-MRI. The study suggests two potential teratogenic mechanisms of ethanol: ethanol-mediated changes in placental permeability and ethanol-induced changes in fetal CBF.

Although MR studies of nonhuman primate FASD models are sparse, a number of studies using invasive methods have been conducted to examine alcohol's effect on the CNS in fetal ethanol exposed monkey fetuses. Multiple exposures of monkey fetuses to alcohol during specific developmental periods cause a reduced number of Purkinje cells in the cerebellum (Bonthius et al. 1996) and neurons in the frontal lobes (Burke et al. 2009). Two recent histological studies in fetal macaque monkeys found that an acute single exposure to alcohol during the third trimester causes widespread neuron apoptosis throughout gray matter regions (Farber et al. 2010) and glial cell (of the oligodentrocyte lineage) apoptosis across white matter regions (Creeley et al. 2013). These disruptions on a cellular level might contribute to the observed changes in neurometabolites observed in MRS studies in both human and animal FASD (Astley et al. 1995; Cortese et al. 2006; Fagerlund et al. 2006; O'Leary-Moore et al. 2008).

Summary and Future Directions

The use of animal models in FASD studies has deepened our understanding of the biological bases of FASD, improving the accuracy of our interpretations of neuroimaging findings in human studies, and provided potential markers for future FASD diagnosis. A significant current goal of many research groups is the development of new noninvasive strategies for early detection of deleterious effects of prenatal ethanol exposure. As Streissguth and colleagues (2004) have noted, the odds ratios of several adverse life outcomes decrease in FASD individuals when therapeutic intervention strategies are initiated early in life. The rationale for this observation has been twofold: CNS plasticity decreases over the first few years of life (Olson et al. 2007), and early diagnosis of FASD is particularly important as it allows "capable caring families to advocate for their children's needs (p. 235)" before establishment of maladaptive behavior (Streissguth et al. 2004). For these reasons, the design of methods for early detection of prenatal ethanol exposure-induced perturbation of normal development remains an important objective in applications of noninvasive neuroimaging tools and animal models of FASD.

As this overview has shown, many laboratories are engaged in research using animal models of FASD. They are implementing studies that vary the timing of ethanol exposure relative to CNS development, along with a diverse array of MRI modalities so they can better understand the consequences of ethanol exposure on anatomical, physio-

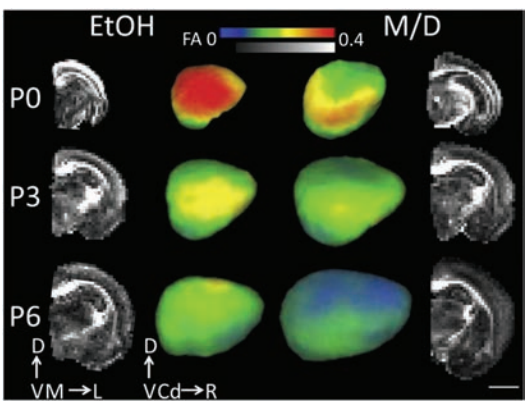

Figure 5 Effect of prenatal ethanol exposure on cerebral cortical fractional anisotropy. The two middle columns of images are laterally facing mid-cortical surface models of one rat at PD 0, PD 3, and PD 6 right hemisphere for each treatment group (ethanol) and maltose/dextrin (M/D), on which cortical fractional anisotropy (FA) at each mid-cortical surface node is projected. The outer columns represent mid-coronal FA maps for the right hemisphere of the same subjects depicted in the middle columns. Cortical FA decreased significantly with age. Additionally, cortical FA was largest, and isocortical volume smallest, in the ethanol group compared with the M/D group. This group difference is most visible in the outer layers of the cortex.

NOTE: Scale bar is 4 mm. D = dorsal, V = ventral, M = medial, L = lateral, Cd = caudal, R = rostral. Figure adapted from Leigland et al. 2013*b*.

logical, and metabolic development (see table). Among the advantages of using noninvasive neuroimaging techniques in animal models is the translatability of findings to clinical studies. In many cases, the biological bases of neuroimaging results obtained in human studies are not well understood. In these cases, parallel neuroimaging experiments with animal models can be performed, and interpretations of the findings can be validated with independent (but often invasive) experimental approaches. Efforts are being made to bridge the MRI findings to histopathological results, which are thought to be the gold standards in and outside FASD research (Jespersen et al. 2012; Leigland et al. 2013*b*; Riddle et al. 2011). In many other cases, studies using MR techniques to monitor CNS development and to examine CNS pathologies (other than FAS/FASD) also can provide valuable perspectives for FASD research. For example, ongoing diffusion MR microscopy efforts have been used to provide a detailed quantitative description of embryonic and early postnatal mouse brain development (Aggarwal et al. 2014; Zhang et al. 2003, 2006). Diffusion anisotropy maps derived from this method show excellent tissue contrast and, as a result, allow visualization of fine microstructural detail of the developing brain. This technique will be useful for investigation of ethanol-induced brain abnormalities in animal models over this age range. In addition, the development of advanced motion correction and imaging reconstruction technique has made in utero MRI examinations possible in humans as well as animals (Fogtmann et al. 2014). Using reconstructed in utero MRI, researchers can delineate human fetal brain tissues (including transient structures present only at early stages of development such as the cortical plate, intermediate zone, ventricular and subventricular zones, etc.) and can plot their growth trajectories (Scott et al. 2011). This close monitoring can help identify fetuses with growth patterns that deviate from the normal trajectory. In turn, their later cognitive outcome could be associated with growth patterns in future studies.

Financial Disclosure

The authors declare that they have no competing financial interests.

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Glossary

Apoptosis: A process of programmed cell death.

Basal ganglia: A part of the brain located at the base of the forebrain and strongly interconnected with several brain regions, including the cerebral cortex, thalamus, and brainstem. The basal ganglia are associated with a variety of functions, including control of voluntary motor movements, procedural learning, and routine behaviors.

Caudate nucleus: One of three basic structures in the brain that make up the basal ganglia and is part of a system that is largely responsible for voluntary movement.

Cerebellum: The part of the brain important for motor control and other functions.

Corpus callosum: A wide, flat bundle of neuronal fibers that connect the left and right hemispheres of the brain.

Ex vivo: Experiments or measurements done on cells or tissues removed from an organism. Ex vivo conditions allow experimentation on cells or tissue under more controlled conditions than in vivo experiments.

Histopathology: The microscopic examination of tissue, indicating disease.

In vivo: Experiments or measurements done on an intact organism.

Morphology: The study of the form and structure of organisms and their specific structural features.

Thalamus: A part of the vertebrate brain made up of two halves deep in the middle of the brain. Among other things, it is involved in relaying sensory and motor signals to the cerebral cortex, and regulating consciousness, sleep, and alertness.

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Prenatal Alcohol Exposure and Cellular Differentiation

A Role for Polycomb and Trithorax Group Proteins in FAS Phenotypes?

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Exposure to alcohol significantly alters the developmental trajectory of progenitor cells and fundamentally compromises tissue formation (i.e., histogenesis). Emerging research suggests that ethanol can impair mammalian development by interfering with the execution of molecular programs governing differentiation. For example, ethanol exposure disrupts cellular migration, changes cell–cell interactions, and alters growth factor signaling pathways. Additionally, ethanol can alter epigenetic mechanisms controlling gene expression. Normally, lineage-specific regulatory factors (i.e., transcription factors) establish the transcriptional networks of each new cell type; the cell's identity then is maintained through epigenetic alterations in the way in which the DNA encoding each gene becomes packaged within the chromatin. Ethanol exposure can induce epigenetic changes that do not induce genetic mutations but nonetheless alter the course of fetal development and result in a large array of patterning defects. Two crucial enzyme complexes—the Polycomb and Trithorax proteins—are central to the epigenetic programs controlling the intricate balance between self-renewal and the execution of cellular differentiation, with diametrically opposed functions. Prenatal ethanol exposure may disrupt the functions of these two enzyme complexes, altering a crucial aspect of mammalian differentiation. Characterizing the involvement of Polycomb and Trithorax group complexes in the etiology of fetal alcohol spectrum disorders will undoubtedly enhance understanding of the role that epigenetic programming plays in this complex disorder. **KEY WORDS: Alcohol exposure; ethanol exposure; prenatal alcohol exposure; prenatal alcohol exposure; fetal alcohol spectrum disorders; fetal alcohol syndrome (FAS); FAS phenotypes; fetal development; epigenetics; epigenetic mechanisms; epigenetic changes; gene expression; developmental programming; transcription; cellular differentiation; Polycomb group proteins; Trithorax group proteins**

Exposure of the developing embryo and fetus to alcohol can have profound adverse effects on physical, behavioral, and cognitive development. The resulting deficits collectively have been termed fetal alcohol spectrum disorders (FASD). They range in severity from mild cognitive deficits to a well-defined syndrome (i.e., fetal alcohol syndrome [FAS]), which is broadly characterized by low birth weight, distinctive craniofacial malformations, smaller-than-normal head size (i.e., microcephaly), and central nervous system dysfunction (Riley et al. 2011). The mechanisms underlying ethanol's harmful effects on development are not yet fully understood. Studies in recent years have indicated that epigenetic mechanisms may play a role in the etiology of FASD. This article describes the proposed roles of epigenetic mechanisms in FASD and cell differentiation in general and introduces two protein complexes that are hypothesized to play central roles in these events.

Role of Epigenetics in Developmental Programming and FASD

Mammalian development consists of a series of carefully orchestrated changes in gene expression that occur as stem or progenitor cells differentiate to form

the tissues and organs making up the growing fetus.[1] Once the identity of each new cell type has been established by lineage-specific transcription factors, this identity is maintained through unique alterations in the way in which the DNA encoding each gene becomes packaged around certain proteins (i.e., the histones) within the chromatin structure of the nucleus (Hemberger et al. 2009). Much like a closed book cannot be read whereas an open book can, the DNA can either be tightly wound up into a structure that silences the encoded genes, or the DNA can be in a relaxed, open, and active state. As development proceeds, the DNA of each cell becomes packaged in a way that is unique to that cell type and thus is programmed to express only a specific set of genes that confer the cell's individual identity and physiological function (Barrero et al. 2010). Three enzymatic mechanisms control the assembly and regulation of chromatin structure: DNA methylation, modification of the histone proteins (i.e., posttranslational histone modification), and ATP-dependent chromatin remodeling (Barrero et al. 2010). These fundamental modifications, which control gene packaging, are passed on to the daughter cells when a cell divides. They are referred to as epigenetic changes because they impart a level of regulation that is above ("epi") the direct genetic modifications of the DNA (Hemberger et al. 2009).

Studies using a diverse range of model organisms have led to the conclusion that epigenetic modifications to the chromatin structure provide a plausible link between exposure to environmental substances that can harm the developing fetus (i.e., teratogens) and lasting alterations in gene expression leading to disease phenotypes. Numerous studies have demonstrated that exposure to ethanol is associated with both genome-wide and gene-specific changes in DNA methylation (Bielawski et al. 2002; Downing et al. 2011; Garro et al. 1991; Haycock and Ramsey 2009; Hicks et al. 2010; Liu et al. 2009; Ouko et al. 2009; Zhou et al. 2011), alterations in posttranslational histone modifications (Kim and Shukla 2005; Pal-Bhadra et al. 2007; Park et al. 2005), and a profound shift in epigenetically sensitive phenotypes (Kaminen-Ahola et al. 2010). Collectively, all of these observations indicate that ethanol can act as a powerful epigenetic disruptor and alter chromatin structure.

Although the mechanisms by which alcohol impacts chromatin structure are not completely understood, recent work suggests that some epigenetic changes result from altered cellular metabolism. For example, Choudhury and colleagues (2010) observed an increase in reactive oxygen species (ROS) within primary rat liver cells (i.e., hepatocytes) treated with ethanol. This increase in ROS was correlated with an increase in a specific modification of histone 3 (i.e., acetylation of histone 3 at lysine 9); moreover, when the cells were treated with cellular antioxidants to eliminate the ROS, these alcohol-induced chromatin modifications were abated (Choudhury et al. 2010). In addition, ethanol exposure has well-documented effects on one-carbon metabolism and the bioavailability of the crucial methyl donor, s-adenosylmethionine (SAMe). Impaired levels of SAMe disrupt the cells' ability to methylate DNA and histones, resulting in compromised epigenetic programming (Zeisel 2011). Interestingly, many of the birth defects observed in FASD also have been noted in studies examining deficiencies in one-carbon metabolism (summarized in Zeisel 2011).

Although alcohol induces several global changes in chromatin structure, many of the associated developmental defects seem to be rooted in gene-specific alterations. A study by Hashimoto-Torii and colleagues (2011) examining global changes in gene transcription within ethanol-exposed samples of brain tissue (i.e., cerebral cortex) reported that of 39,000 candidate messenger RNAs (mRNAs) assessed, only 636 transcripts were differentially expressed. Other researchers have identified alcohol-induced alterations in the expression of only a small number of key developmental regulators, including several transcription factors known as *HOX* factors, which play crucial roles in directing organ patterning and morphogenesis (Godin et al. 2011; Mo et al. 2012; Rifas et al. 1997; Vangipuram and Lyman 2012). In rodent models, these alterations have been associated with neural patterning defects and the development of abnormalities in structures of the head and face (i.e., craniofacial dysmorphogenesis), reminiscent of those observed in clinical studies of FASD (Parnell et al. 2009; Rifas et al. 1997). However, these alcohol-induced alterations in gene expression often are limited to a specific tissue type and arise only when ethanol exposure occurs during select developmental windows (Godin et al. 2011; Kim et al. 2010; Mo et al. 2012; Parnell et al. 2009). These observations suggest that the molecular machinery involved in epigenetic programming also may be disrupted by ethanol exposure and, as a consequence, key epigenetic cues regulating development are not properly established.

Epigenetic Control and Developmental Programming of Differentiation

Of the three classes of epigenetic modifications, posttranslational modification of histone proteins undoubtedly is the most complex. Posttranslational enzymatic modifications, such as acetylation, methylation, phosphorylation, and ubiquitination (which have been studied most extensively), work together to produce a combinatorial "histone code" that serves to regulate cell-lineage–specific patterns of chromatin structure throughout development (Fisher and Fisher 2011). Within the unique transcriptional environment of embryonic stem cells, several developmentally

[1] Stem cells still have the ability to differentiate into any type of specialized cell (e.g., nerve, blood, or muscle cells); therefore, they are called pluripotent. Progenitor cells already are more specialized and therefore committed to a certain type of tissue; for example, neural progenitor cells can develop into different types of nerve cells or supporting brain cells (i.e., glial cells) but can no longer differentiate into muscle or blood cells. Thus, these progenitor cells are multipotent but not pluripotent.

crucial genes are marked in a coordinated fashion with both activating and repressive histone modifications (Bernstein et al. 2006; Jiang et al. 2011; Lim et al. 2009). Specifically, histone 3 (around which DNA sequences are wrapped) is modified by the addition of three methyl groups to the fourth lysine residue (i.e., histone 3 lysine 4 trimethylation), which typically is associated with gene activation, as well as by trimethylation of lysine 27, which has repressive effects (see figure 1A). The DNA sequences wrapped around these uniquely marked histones are termed bivalent domains and generally encode transcription factors directing tissue-specific programs of differentiation (Fisher and Fischer 2011). This same distinctive signature is found, albeit less frequently, in placental, neuronal, and other tissue-specific progenitor cell types (Lim et al. 2009; Rugg-Gunn et al. 2010). These bivalently marked genes generally are not expressed but are thought to be "primed" for either rapid activation or silencing during differentiation. Once a progenitor cell's fate has been established by lineage-specific transcription factor networks, the cell's transcriptional memory is maintained by removing one of the coexisting modifications and leaving only the modification indicative of the active or silent state in place. Importantly, many bivalently marked genes are disrupted in prenatal models of alcohol exposure, which potentially may explain the constellation of effects observed in FASD. For example, in a neural stem cell model ethanol exposure alters both histone 3 lysine 4 and lysine 27 trimethylation (Veazey et al. 2013). Understanding the mechanistic basis of these epigenetic defects is crucial to deciphering the developmental origins of FASD.

Seminal studies using the fruit fly *Drosophila melanogaster* in the late 1970s to early 1980s revealed the existence of two large multiprotein complexes with diametrically opposite roles in the regulation of gene expression: the Polycomb group (PcG) and Trithorax group (TrxG) (Lewis 1978; Poux et al. 2002; Schuettengruber et al. 2007). These two developmentally crucial enzyme complexes function at the hub of mammalian development; by binding to the regulatory regions of bivalent genes, they regulate the intricate balance between self-renewal of stem and progenitor cells and the execution of cellular differentiation. As differentiation progresses, these regulatory regions "commit" to one of these two protein complexes and become occupied exclusively by either the PcG or TrxG proteins. This commitment occurs in a cell-lineage–dependent manner, and

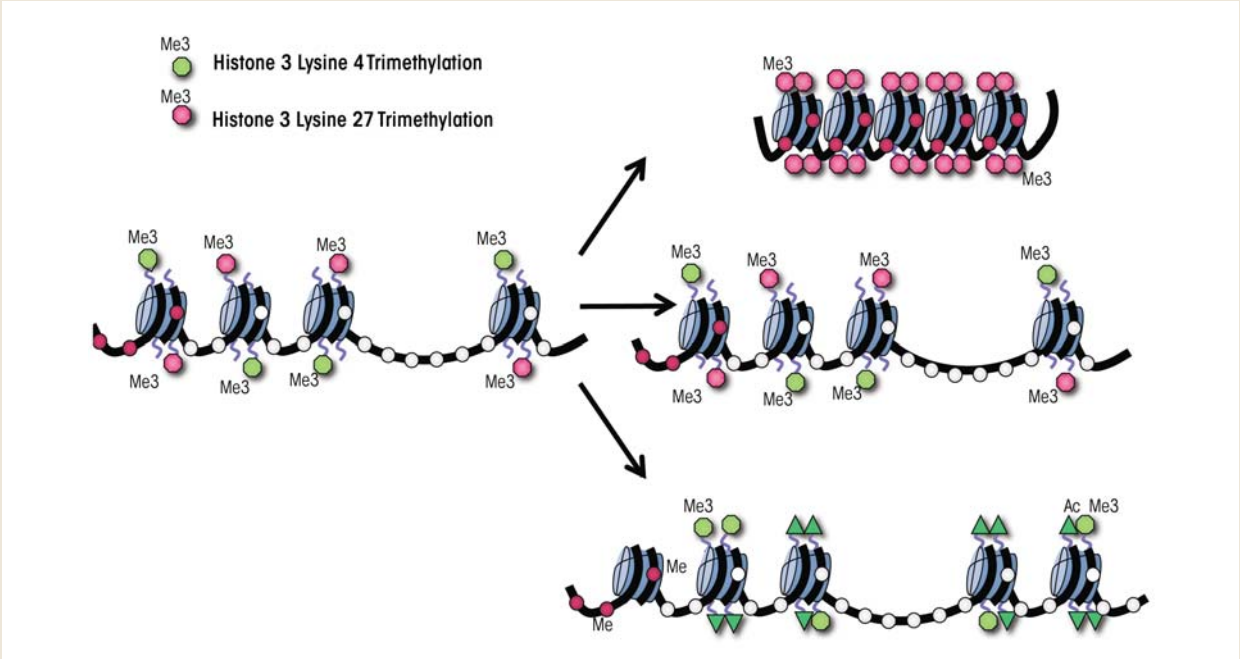

Figure 1A Bivalent state of the DNA in mammalian cells and its resolution during differentiation. In stem or progenitor cells, numerous developmentally relevant genes encoding factors that drive lineage-specific patterning are simultaneously marked with both activating and repressive histone modifications. This bivalent chromatin signature is thought to silence lineage-specifying genes through histone 3 lysine 27 trimethylation (H3K27me3) while at the same time poising them for activation during differentiation through the presence of histone 3 lysine 4 trimethylation (H3K4me3). As differentiation progresses, these domains can either adopt a silent conformation (top), become transcriptionally active (bottom), or persist into the next progenitor cell type (middle).

as a result the chromatin structure of these bivalent genes becomes fixed in either an active or a silent state. Any defects in this delicate balancing act, particularly during the differentiation towards a neural lineage, results in developmental defects and causes disease. Despite their fundamental importance to the processes of epigenetic programming and mammalian development, however, the roles of PcG and TrxG proteins in the etiology of FASD to date have not been examined.

PcG Proteins

The PcG proteins and the genes encoding them originally were discovered over 30 years ago as key regulators of the processes that specify which end of the embryo forms the head and which the rear during the development of Drosophila (Lewis 1978). Since then, researchers have found that these gene families encode essential regulators governing mammalian processes of cellular determination and lineage-specific patterns of differentiation. In mammals, two major PcG complexes have been characterized that modify chromatin structure; these are called Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2). Each complex is composed of several proteins with different biochemical functions, many of which are not well understood (see figure 2). PRC1 acts by mediating the ubiquitination of the 119th lysine residue of histone H2A; this is achieved by two of the PRC1 proteins called ring finger protein 1A and 1B (RING1A and RING1B) (Wang et al. 2004). This posttranslational modification pushes the local chromatin structure towards a transcriptionally repressive state and its proper establishment is essential to the coordinated silencing of genes throughout mammalian development (Boyer et al. 2006; Wang et al. 2004). In embryonic stem cells, histone ubiquitination stabilizes the presence of an enzyme called RNA polymerase II (which is required for gene expression) at bivalent chromatin domains and is crucial for maintaining the pluripotent state of undifferentiated cells (Ku et al. 2008).

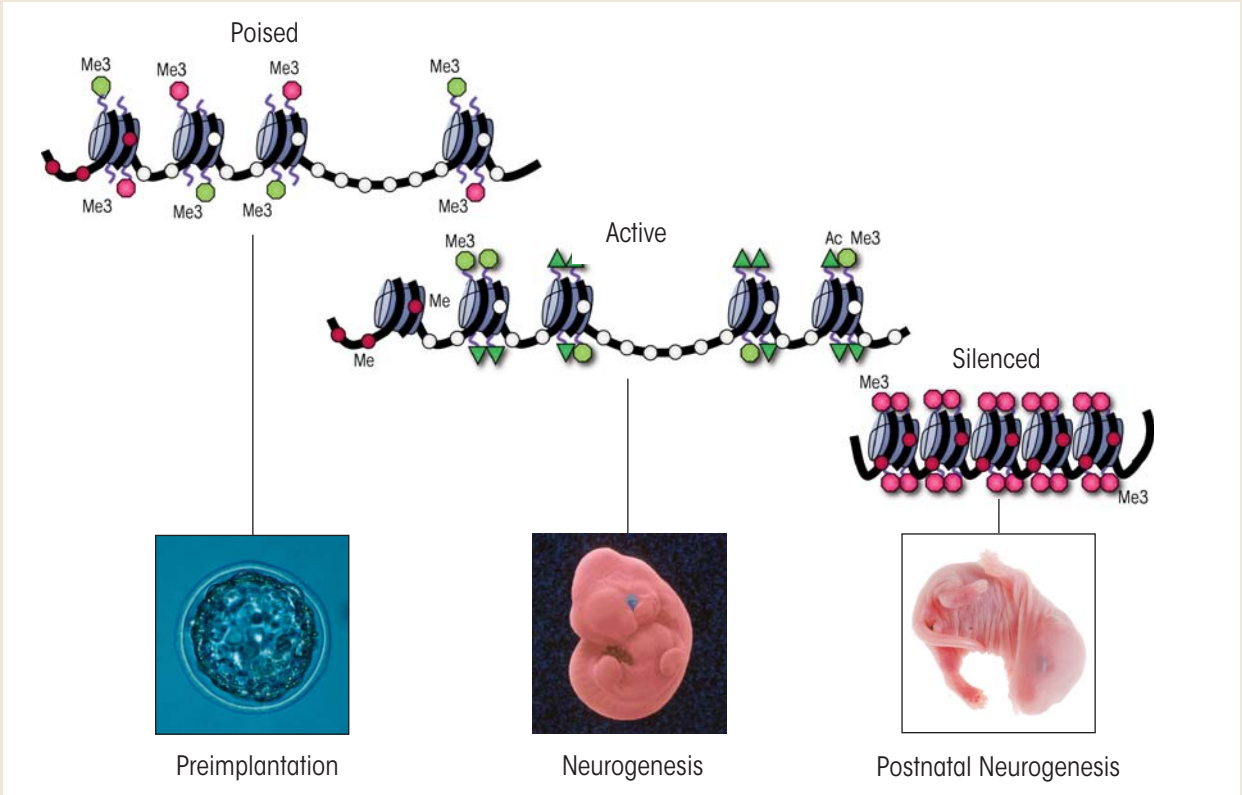

Figure 1B During development of the nervous system, many genes controlling neural patterning are held in a poised or bivalent conformation during early embryogenesis, resolve towards the active conformation during neural patterning, and are silenced during postnatal life. Repression (i.e., trimethylation of histone 3 lysine 27 [H3K27me3]) is imposed by the polycomb group proteins (PcG) (small red circles), whereas activation H3K4me3 is imparted by the mammalian homologues of the trithorax group proteins (TrxG) (green triangles). Correct biochemical function of these proteins and the coordination of the marks they impart are essential to mammalian neurogenesis.

PRC2 has similar repressive properties to PRC1 and also is an essential regulator of cellular differentiation. It facilitates the silencing of developmentally crucial genes through mono-, di-, and trimethylation of the histone 3 lysine 27 and trimethylation of histone 3 lysine 9 (Cao et al. 2002; Czermin et al. 2002), both of which repress gene expression. Together, the methylation of these two lysine residues promotes the generation of facultative heterochromatin[2] and mediates a transcriptionally silent state.

Adding an additional layer of complexity, PRC2 associates with the mammalian enzymes responsible for DNA methylation (i.e., DNA methyltransferase complexes); this association aids in the ability of PRC2 complexes to repress their target loci (Viré et al. 2006). This physical interaction suggests that the PcG complexes and the DNA methyltransferases act together to maintain the epigenetic memory of chromatin states throughout differentiation. Proper functioning of this gene family and their interacting proteins is essential for the execution of cell-specific differentiation programs and proper lineage specification (Pasini et al. 2007).

[2] The term "facultative heterochromatin" refers to gene-rich regions of the genome that are silent, but which can dynamically cycle into periods of transcriptional activity. For example, a chromosome region containing a gene that is active only during late development will be silent during early development, become transcriptionally active during late development, and then return to a silent state for the adult stage of life. Conversely, other genomic regions are held perpetually in a tightly compact, silent state; these regions are known as constitutive heterochromatin.

TrxG Proteins

In fruit flies, maternal transcription factors that were included in the egg cells and which are distributed unevenly throughout the developing embryo shape gene expression in the early embryo. The levels of these transcription factors diminish over time, and once they disappear from the developing embryo, the memory of which genes were active in a given cell is propagated through the action of the TrxG proteins (Lewis 1978; Poux et al. 2002). These proteins also have been identified in mammals, where they have been implicated in fundamental epigenetic and cellular processes, including X-chromosome inactivation, genomic imprinting, stress response, programmed cell death (i.e., apoptosis), development

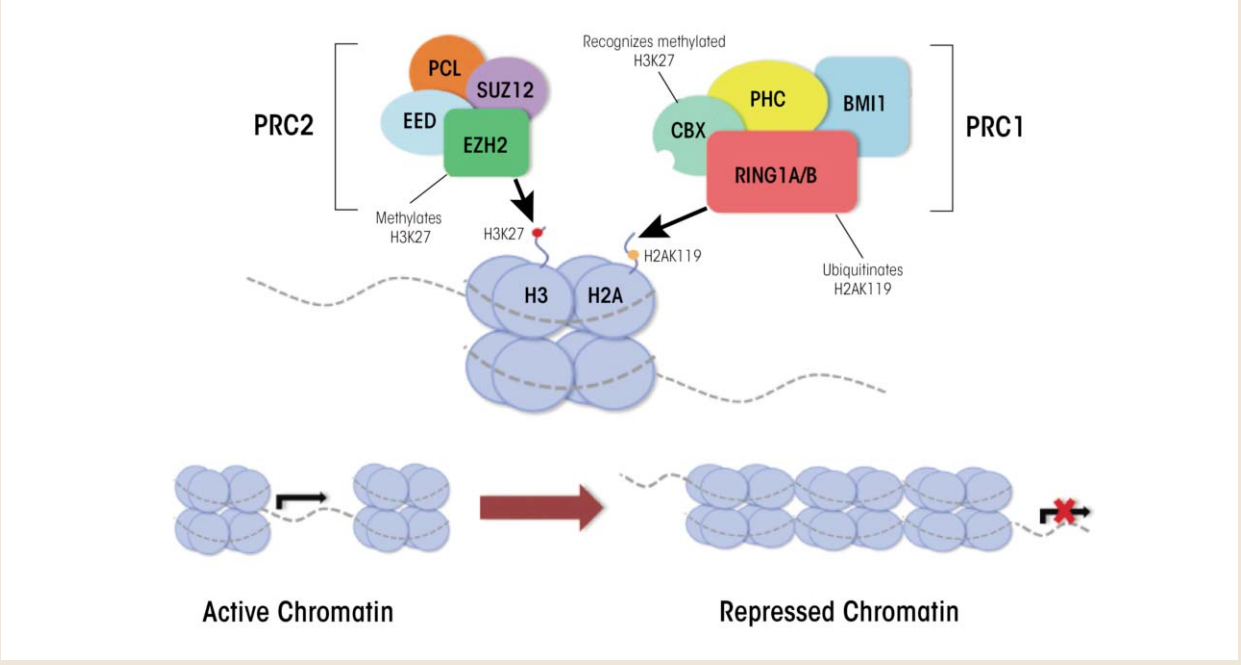

Figure 2A Transcriptional regulation by the Polycomb and Trithorax complexes. Polycomb repressive complex 1 (PRC1) consists of four core proteins including: polyhomeotic homolog (PHC), ring finger protein 1A or 1B (RING1A or RING1B), B-lymphoma Mo-MLV insertion region 1 homolog (BMI1), and chromobox homolog (CBX). The RING1A/RING1B subunits are the catalytic engine of the PRC1 complex and carry out ubiquitination of histone 2A at lysine 119 (H2AK119ub). PRC2 consists of four core proteins including: embryonic ectoderm development (EED), enhancer of zeste 2 (EZH2), suppressor of zeste 12 (SUZ12), and polycomb like (PCL). EZH2 serves as the catalytic subunit of PRC2 and trimethylates lysine 27 on histone 3 (H3K27me3). Current models suggest that H3K27me3 generated by PRC2 facilitates compaction of chromatin leading to the repression of gene expression. Subsequently, the CBX subunit of the PRC1 complex recognizes H3K27me3, and the RING1A/RING1B subunits of PRC1 ubiquitinate H2AK119 to facilitate the maintenance of the repressed state.

of tumors (i.e., tumorigenesis), cell proliferation, and embryonic stem cell renewal. However, compared with the PRC1 and PRC2 complexes, very little information exists on individual TrxG proteins or their biochemical functions (Schuettengruber et al. 2007). It is known that TrxG proteins function as multiprotein complexes that mediate the trimethylation of histone 3 lysine 4 (H3K4me3) and which have been conserved across different species (Jiang et al. 2011). In mammalian cells the TrxG complex is formed by a core group of structural proteins that combine with at least one of six interchangeable histone methyltransferases.

The main core of TrxG complexes is composed of four proteins called WD40 repeat domain 5 (WDR5), retinoblastoma binding protein 5 (RbBP5), dosage compensation–related protein 30 (Dpy30), and absent, small, or homeotic-like protein (Ash2L) (see figure 3). WDR5 recognizes histone 3 molecules that are methylated at lysine 4 and allows the methyltransferase in the TrxG complex to bind to this region and add another methyl group; thus, WDR5 is an essential regulator of global H3K4 trimethylation (Wysocka et al. 2005). RbB5 is necessary for proper differentiation of embryonic stem cells into neural progenitor cells and, together with Dpy30, also is essential for regulating global levels of H3K4 trimethylation (Jiang et al. 2011).

The TrxG core interacts with a group of interchangeable H3K4 methyltransferases, including some called mixed lineage leukemia (MLL) proteins (i.e., MLL1, MLL2, MLL3, and MLL4) and proteins called SET1A and SET1B (Jiang et al. 2011; Steward et al. 2006). MLL1 initially was discovered in cells of patients with different types of leukemia (i.e., acute lymphoid and acute myeloid leukemia). It is thought to promote cell-specific patterns of gene expression by regulating global and gene-specific H3K4 methylation during early embryonic development (Yu et al. 1995), because mice in which the corresponding mouse gene (*MLL1*) has been eliminated, or knocked out, show alterations in H3K4 methylation. In contrast, knockout of the *MLL2* gene in mouse embryonic stem cells leads to skewed differentiation but no concrete alterations to H3K4 methylation (Lubitz et al. 2007). For the remaining methyltransferases (i.e., MLL3, MLL4, and SET1A/1B), little is known except that they are involved in H3K4 methylation. Deletion of any one of these other methyltransferases seems to have

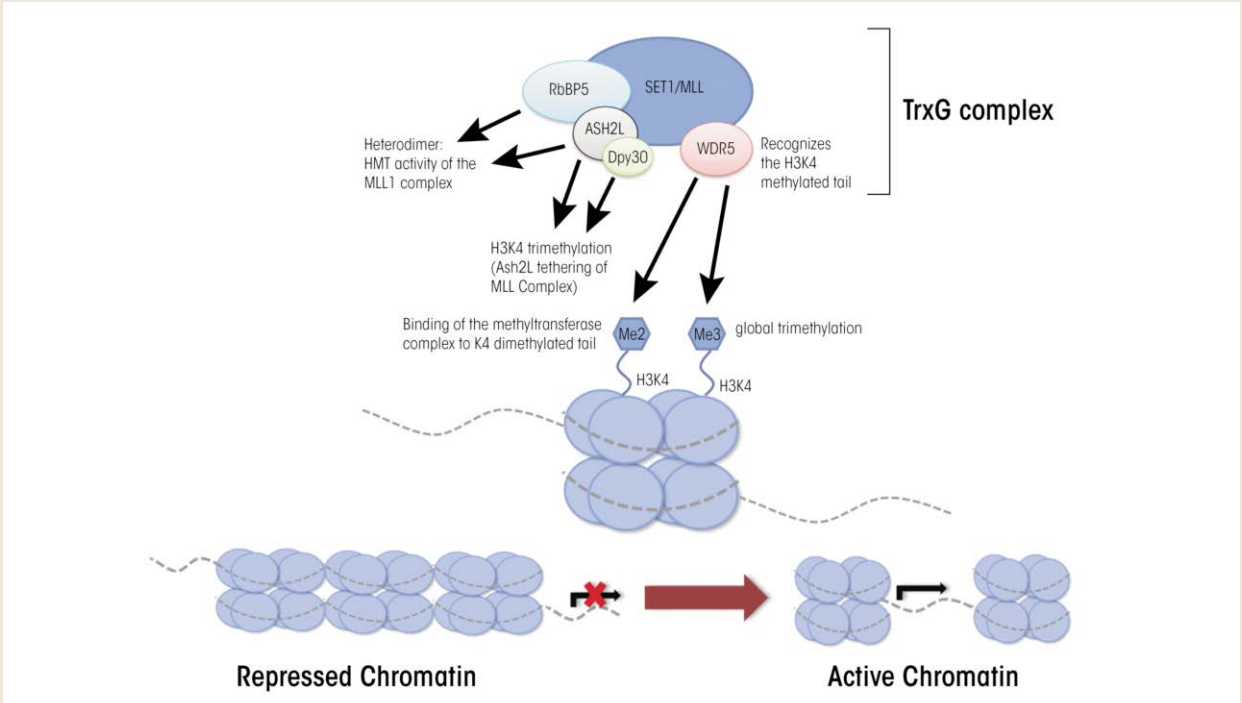

Figure 3 Trithorax (TrxG) proteins function as a conserved multi-component complex that regulates the trimethylation of histone 3 lysine 4 (H3K4me3). The four core structural components of the TrxG complex are: WD40 repeat domain 5 (WDR5), retinoblastoma binding protein 5 (RbBP5), dosage compensation-related protein 30 (Dpy30), and absent, small, or homeotic-like (Ash2L). These proteins serve as a scaffold to regulate the biological activity of the H3K4 methyltransferase family of enzymes, which include mixed-lineage leukemia (MLL) proteins MLL1, MLL2, MLL3, and MLL4, as well as SET1A and SET1B.

only minimal effects on global levels of H3K4 methylation, likely because the remaining MLL family members can substitute for the deleted ones (Jiang et al. 2011). Thus, although researchers have made progress in clarifying the roles of TrxG proteins, much remains unknown regarding the temporal and tissue-specific regulatory events these proteins promote.

Role of PcG and TrxG in the Etiology of FASD

Postmortem studies of children that succumbed to FAS revealed groups of poorly differentiated neuronal and glial cells at abnormal sites within the brain, suggesting large-scale problems with cellular proliferation and differentiation resulting from prenatal alcohol exposure (Swayze et al. 1997). Furthermore, studies using animal models have demonstrated reduced brain size and abnormal migration of neural cells in mice exposed to ethanol in utero (Godin et al. 2010; Parnell et al. 2009). Collectively, these observations indicate that alcohol impairs the cellular processes of neuronal differentiation and migration during fetal development. In support of this conclusion, studies using human and rodent neurosphere cultures have demonstrated that treatment with ethanol increases neurosphere size, skews the developmental potential of neural progenitor cells, and fundamentally alters the neuronal differentiation program (Roitbak et al. 2011; Vangipuram and Lyman 2012). However, the specific molecular mechanisms by which ethanol disrupts the cellular processes governing differentiation remain poorly defined. Recent studies examining the consequences of ethanol exposure during embryonic stem cell differentiation demonstrate a delay in the ability of exposed cells to silence regulatory factors promoting pluripotency, including the transcription factors OCT4, NANOG, and SOX2 (Arzumnayan et al. 2009). These studies strongly suggest that ethanol interferes with the ability of differentiating cells to recruit epigenetic modifiers to genes playing key roles in development and to execute the molecular programs governing cellular differentiation.

During early mammalian development, approximately 2,000 genes are bivalently marked as described earlier, and these marks progressively resolve towards the lineage-specific patterns of chromatin organization characterizing each unique cell type (Rugg-Gunn et al. 2010). As development proceeds, many precursor cell types maintain a subset of developmentally critical genes in this conformation as well push new groups of cellular factors into a bivalent state. For example, in pluripotent embryonic stem cells (which can differentiate into any cell type) the neural precursor genes *Dlx2, Hand1, Msx2, Nestin, Nkx2.1, Nkx2.2, Olig2, Pax6,* and *Sox1* all are bivalently marked, whereas in multipotent, neural precursor cells (which only can develop further into different types of neurons) only *Dlx2* and *Pax6* maintain this conformation. Interestingly, two genes encoding marker proteins that are found only in a type of glial cell called astrocyte (i.e., myelin basic protein [MBP] and glial fibrillary acidic protein [GFAP]) establish novel bivalent domains so that these genes can be kept in an active or inactive state, depending on whether they will become nerve cells or astrocytes (Golebiewska et al. 2009). Proper functioning of the TrxG complexes is indispensable to converting these bivalent loci into the actively transcribed state required for the induction of nerve cell formation (i.e., neurogenesis) (Huang et al. 2007; Jiang et al. 2011; Lim et al. 2009). Similarly, PcG complexes are necessary to silence the myriad of developmental regulators that would be required if the cells would differentiate into other cell types; thus, these complexes also help ensure that lineage-specific patterns of gene expression arise (Pereira et al. 2010). By propagating the transcriptional memory established by lineage-specific transcription factor networks, the TrxG and PcG complexes cooperatively regulate the balance between stem cell renewal and lineage differentiation.

Importantly, the expression of many of these factors that are bivalently marked and regulated by PcG and TrxG is disrupted in various models of prenatal alcohol exposure; moreover, this disruption is associated with profound errors in neuronal patterning. For example, alcohol suppresses the activation of two neural precursor genes—*Msx2* and *Pax6*—leading to craniofacial abnormalities and excessive differentiation of glutamatergic neurons, respectively (Kim et al. 2010; Mo et al. 2012; Rifas et al. 1997). Similarly, both the expression and localization of *Nkx2.1* and *Olig2* are diminished by alcohol, potentially disrupting the balance between excitation and inhibition in the cerebral cortex after birth (Godin et al. 2011). Finally, recent studies by Taléns-Visconti and colleagues (2011) have demonstrated that ethanol affects the proliferation of neural progenitor cells and markedly reduces their potential to differentiate into mature neurons, astrocytes, and another type of glial cell called oligodendrocytes. Given this broad-spectrum impediment to nearly every neuronal developmental fate, it is possible that the observed impact of ethanol on the overall architecture and size of the brain in FAS children stems from effects on some aspect of PcG/TrxG regulation of neural precursor differentiation. Using a neurosphere model of differentiation, Mo and colleagues (2012) recently demonstrated that expression of the *Pax6* gene at a site other than where it usually is expressed could ameliorate the impact of ethanol on cell proliferation and neurogenesis. These results suggest that within a limited scope it may be possible to reverse alcohol's effects on developmental programs.

Conclusions

One of the most difficult aspects in the study of FASDs has been trying to explain the wide range of severity and enormous variation in FASD-associated

birth defects. The process of organ formation is initiated during the early stages of embryonic development, and different rudimentary organ systems are formed and grow during unique developmental windows (Zorn and Wells 2009). Each organ system cycles between periods of intense growth and steady-state maintenance. The periods of growth are characterized by carefully orchestrated changes in DNA methylation and chromatin structure as differentiating cells are programmed with their epigenetic identity (Zhou et al. 2011). Studies using animal models analyzing the correlation of ethanol exposure at varying developmental time points with major periods of tissue growth strongly indicate that different tissues primarily are susceptible to ethanol-induced teratogenesis during specific developmental windows (Becker et al. 1996). Given the demonstrated ability of alcohol to alter DNA methylation and chromatin structure, it is likely that in organ systems which enter or are in a period of active epigenetic programming, ethanol exposure induces lasting epigenetic lesions that persist throughout organogenesis, whereas non-developing systems remain largely refractory to alcohol's effects. Thus, the epigenetic errors resulting from alcohol exposure can vary greatly depending on the specific timing and dose of alcohol exposure, which can explain the wide diversity in severity and range of birth defects that characterize FASD (Becker et al. 1996).

Since their discovery, the PcG and TrxG protein complexes have been identified in numerous disease contexts, including cellular transformation of normal cells into tumor cells as well as structural defects and mental illness (Huang et al. 2007; Varambally et al. 2002; Yu et al. 1995). These studies have demonstrated that a molecular event or teratogen (e.g., ethanol) that alters PcG/ TrxG programming within even a few neural progenitor stem cells during fetal growth can disproportionately influence subsequent brain development and potentially impart severe neurological birth defects (Boyer et al. 2006, Hirabayashi and Gotch. 2010). A complete characterization of the involvement of PcG and TrxG complexes in the etiology of FASD will undoubtedly aid in understanding the role of epigenetic programming in this complex disorder. ■

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In Utero Alcohol Exposure, Epigenetic Changes, and Their Consequences

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Exposure to alcohol has serious consequences for the developing fetus, leading to a range of conditions collectively known as fetal alcohol spectrum disorders (FASD). Most importantly, alcohol exposure affects the development of the brain during critical periods of differentiation and growth, leading to cognitive and behavioral deficits. The molecular mechanisms and processes underlying the teratogenic effects of alcohol exposure remain poorly understood and are complex, because the specific effects depend on the timing, amount, and duration of exposure as well as genetic susceptibility. Accumulating evidence from studies on DNA methylation and histone modification that affect chromatin structure, as well as on the role of microRNAs in regulating mRNA levels supports the contribution of epigenetic mechanisms to the development of FASD. These epigenetic effects are difficult to study, however, because they often are cell-type specific and transient in nature. Rodent models play an important role in FASD research. Although recent studies using these models have yielded some insight into epigenetic mechanisms affecting brain development, they have generated more questions than they have provided definitive answers. Researchers are just beginning to explore the intertwined roles of different epigenetic mechanisms in neurogenesis and how this process is affected by exposure to alcohol, causing FASD. **KEY WORDS: Prenatal alcohol exposure; fetal alcohol spectrum disorders; epigenetics; epigenetic mechanisms; epigenetic modifications; brain development; cognitive deficits; behavioral deficits; DNA methylation; histone modification; chromatin; microRNAs; rodent models**

Alcohol exposure of the developing embryo and fetus in utero can have a wide range of detrimental effects collectively referred to as fetal alcohol spectrum disorders (FASD). Researchers are intensively investigating the mechanisms that may contribute to alcohol's effects on the developing organism and to the resulting consequences, particularly with respect to the cognitive and behavioral deficits associated with FASD. These studies have yielded increasing evidence that epigenetic mechanisms play an important role in these processes. This article reviews the current knowledge regarding the contributions of epigenetic modifications to the manifestations of FASD, much of which has been obtained using rodent models in which the timing, frequency, duration, and amount of alcohol exposure can be tightly controlled. This discussion also touches on the concepts of developmental reprogramming, the role of preconception alcohol exposure, and transgenerational transmission of the effects of alcohol exposure.

FASD

FASD can be associated with a variety of symptoms that differ widely in severity depending on the specific conditions of alcohol exposure. The most severe outcome is fetal alcohol syndrome (FAS), which can manifest variably with diverse combinations of craniofacial, growth, central nervous system (CNS), and neurobehavioral abnormalities (Jones et al. 1973; Sampson et al. 1997). Associated psychosocial problems include learning difficulties,

attention deficit–hyperactivity disorder (ADHD), and mental retardation (Burd et al. 2003; O'Leary 2004). Given that alcohol consumption is voluntary, FASD is said to be the most preventable cause of birth defects and mental retardation. FASD is a global health concern, and worldwide approximately 1 to 3 per 1,000 births is thought to be suffering from FAS. In the United States, FAS prevalence ranges between 0.5 and 2.0 per 1,000 live births (Abel 1995; May and Gossage 2001). The highest rates of FAS have been reported in mixed-ancestry communities in the Western Cape of South Africa, where between 68.0 and 89.2 per 1,000 school-age children display FAS symptoms (May et al. 2007).

Between 5 and 10 percent of offspring who have been exposed to alcohol prenatally display alcohol-related developmental anomalies (Abel 1995), with the severity of the outcome determined by the dose, timing, and duration of exposure (Padmanabhan and Hameed 1988; Pierce and West 1986; Sulik 1984). However, the proportion of affected offspring may be considerably higher in unfavorable circumstances, including instances of malnutrition of the mother and thus, the fetus. The genetic makeup of both mother and fetus, in conjunction with other factors (e.g., gender, diet, and social environment), also plays an important role in the manifestation of FASD (Chernoff 1980; Ogawa et al. 2005).

The effects of prenatal alcohol exposure are more similar in identical (i.e., monozygotic) twins than in fraternal (i.e., dizygotic) twins, suggesting a heritable component (Abel 1988; Chasnoff 1985; Streissguth and Dehaene 1993). Genetic studies have shown that different variants of the genes encoding various alcohol-metabolizing enzymes—such as alcohol dehydrogenases (ADHs), aldehyde dehydrogenases (ALDHs), and cytochrome P450 2E1 (CYP2E1)—in the mother and their offspring can affect alcohol metabolism and contribute to subsequent alcohol-related damage (Gemma et al. 2007; Warren and Li 2005). For example, variants at the ADH1B locus that result in an altered amino acid sequence and function of the encoded enzyme can influence the severity of the adverse effects on the developing fetus (i.e., teratogenesis) in different ethnic populations (for a review see Ramsay 2010). However, to date few studies have supported a role for genetics in the development of FASD.

Rodent models have provided a valuable tool for investigating genetic influences on the observable outcomes (i.e., phenotypes) associated with FASD. For example, the effects of in utero alcohol exposure differ between inbred and selectively bred mice. These findings highlight the contribution of a genetic predisposition to the susceptibility to the detrimental effects of prenatal ethanol exposure and provide additional support for the importance of genetic factors in the development of FASD (Boehm et al. 1997; Gilliam et al. 1989; Ogawa et al. 2005).

Although studies have investigated the genetic susceptibility to FASD, the underlying cause(s) of these disorders still remains unclear. The wide range of clinical features observed in people affected by in utero alcohol exposure underlines the importance of investigating the mechanisms of alcohol-related teratogenesis at a molecular level. Because FASD is a developmental abnormality, disruptions in normal cellular differentiation driven by changes in gene expression that in turn are regulated by epigenetic mechanisms are most likely involved in FASD pathogenesis.

Epigenetic Modifications

The term epigenetics, first defined by Waddington in 1942 (as reprinted in Waddington 2012), refers to the changes in gene expression that occur without changes in the DNA sequence itself. Epigenetics plays a vital role in regulating key developmental events, allowing for tissue-specific gene expression, genomic imprinting,[1] and stem-cell maintenance. Tissue-specific gene expression patterns are established and maintained through two mechanisms; structural chromatin modifications (i.e., DNA methylation and histone modifications) and RNA interactions (i.e., the actions of non-coding RNAs [ncRNAs]). In eukaryotes, the genome is present in the cell nucleus in the form of chromatin—a DNA–protein complex that packages DNA into a highly condensed form. The structural building blocks of chromatin are the nucleosomes, each of which consists of 147 base pairs of DNA wrapped around a core of 8 histone proteins (Ooi and Henikoff 2007). The octamer core comprises two copies each of histone proteins H2A, H2B, H3, and H4. Moreover, the nucleosomes are connected with each other by a linker histone H1 that offers stability to the packaged structure. Modifications of the chromatin structure affect the first step of gene expression (i.e., transcription). ncRNAs, on the other hand, act at the posttranscriptional level.

Chromatin Remodeling

DNA Modifications. Both DNA and protein components of the nucleosome are subject to a variety of modifications that can influence chromatin conformation and accessibility. The best-characterized epigenetic mark, DNA methylation, involves the covalent addition of a methyl (CH_3) group to one of the four DNA nucleotides (i.e., cytosine [C]) to form 5-methylcytosine (5mC). In eukaryotes, methylation usually affects C that are followed by the nucleotide guanine (G) (i.e., that are part of a CpG dinucleotide) (Rodenhiser and Mann, 2006). At these sites, enzymes called DNA methyltransferases (DNMTs) mediate the methylation

[1] Genomic imprinting is a genetic phenomenon in which only a gene copy inherited from one parent is expressed in the offspring. Each person inherits two copies of each gene, one inherited from the mother and one inherited from the father. In general, both of these gene copies can be active in the offspring. For some genes, however, one gene copy is "shut off" by genomic imprinting, so that only the non-imprinted copy remains active. For certain genes, the nonimprinted, active copy is always the one inherited from the mother, whereas for other genes the nonimprinted, active copy is always the one inherited from the father.

of C residues, thereby acting as critical modulators of fetal development (Li et al. 1992). For these DNA methylation reactions, DNMTs use methyl groups produced by a sequence of reactions known as the folate pathway (Friso et al. 2002). Generally, DNA methylation is associated with a condensed chromatin conformation, which effectively silences gene expression because the enzymes needed for transcription cannot access the DNA.

More recent studies have found that 5mC can be further modified by enzymes called ten-eleven translocation (Tet) proteins, in a process referred to as iterative oxidation. This results in the formation of several reaction products (i.e., derivatives), including 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (Ito et al. 2011; Tahiliani et al. 2009). Although the role of these methylation derivatives still remains unclear (Branco et al. 2012) they seem to serve different functions than 5mC. Thus, the conversion of 5mC to 5hmC has been implicated in active DNA demethylation (Wu and Zhang, 2010). Furthermore, whereas 5mC typically is found in regions regulating the expression of specific genes (i.e., in promoters), 5hmC is associated with the bodies of the affected genes or with promoters of developmental regulatory genes (Wu et al. 2011). Finally, 5hmC appears to play an important role in reprogramming the paternal genome following fertilisation (Hackett and Surani 2013). (Reprogramming will be discussed in the following section.)

Histone Modifications. The histones making up the core of the nucleosome have unstructured N-terminal tails that protrude from the nucleosome and which are subject to modifications. Histone modifications are varied and include acetylation, methylation, phosphorylation, ubiquitinylation, ADP-ribosylation, and sumoylation at specified residues (for a review, see Kouzarides 2007). Importantly, these modifications are dynamic—that is, they can be removed again by specific enzymes.

These histone modifications, together with DNA methylation, influence chromatin structure and have a profound influence on gene regulation. Both of these types of epigenetic modifications work together to remodel the chromatin and partition the genome into two different functional domains—transcriptionally active regions collectively known as euchromatin and transcriptionally inactive regions collectively called heterochromatin. Euchromatic regions are modified to allow an open conformation, rendering the regions accessible to cellular proteins favoring transcription. In contrast, heterochromatic regions, such as the ends of chromosomes (i.e., telomeres) and regions around the center of the chromosome (i.e., pericentric regions), generally exhibit a closed conformation that limits interactions between the DNA and cellular proteins, thereby silencing gene activity (for a review, see Schneider and Grosschedl 2007). Additionally, chromatin structure, and thus gene expression, is influenced by the specific combination of histone variants in a nucleosome, the spacing between nucleosomes (i.e., nucleosome occupancy), and the position of each nucleosome within the nucleus (i.e., nuclear architecture) (Cairns 2009).

Developmental Reprogramming

Epigenetic reprogramming is a process that involves the erasure and then re-establishment of chromatin modifications during mammalian development. It serves to erase random changes in epigenetic marks (i.e., epimutations) that have occurred in the germ cells (i.e., gametes) and to restore the ability of the fertilized egg cell (i.e., zygote) to develop into all the different cell types and tissues (Reik et al. 2001). Epigenetic modifications are modulated in a temporal and spatial manner and act as reversible switches of gene expression that can lock genes into active or repressed states. In addition, these modifications allow the zygote to give rise to the cellular lineages that will form the embryo. Reprogramming occurs in two phases during in utero development, one shortly after fertilization and the other in the developing gametes of the fetus. The first phase takes place after fertilization in the preimplantation embryo (i.e., the blastocyst). During this phase, embryonic epigenetic patterns are re-established in a lineage-specific manner in the inner cell mass of the blastocyst (figure 1). The second phase occurs in the gametes, where rapid genome-wide demethylation is initiated to erase existing parental methylation patterns, followed by re-establishment of epigenetic marks in a sex-specific manner (Reik et al. 2001).

Researchers recently have begun to investigate epigenetic mechanisms as key contributors to the development of FASD. This research was prompted by the observation that periods of increased vulnerability to in utero alcohol exposure coincide with those of reprogramming events. In addition, evidence suggests that environmental factors, and specifically alcohol, are able to alter epigenetic modifications. This provides a link between the genotype, environment, and disease.

Alcohol and Biological Pathways

As mentioned previously, DNA methylation reactions rely on the folate pathway to supply the necessary methyl groups. Excessive alcohol exposure is known to interfere with normal folate metabolism and reduce its bioavailability (Halsted and Medici 2012). Folate is required as a coenzyme to supply methyl groups needed for the formation of a compound called S-adenosylmethionine (SAMe), which in turn participates in reactions in which the methyl group is transferred to another molecule (i.e., transmethylation reactions). In the folate-dependent pathway, the enzyme methionine synthase (MS), which requires vitamin

B12 to function properly, is responsible for transferring the methyl group contained within the 5-methyl-tetrahyrofolate compound to homocysteine, which ultimately generates methionine (Friso et al. 2002). The methionine is converted to SAMe by methionine adenosyltransferase (MAT), and the SAMe then is used for the methylation of DNA. As early as 1974, research on alcohol-fed rats described reduced MS activity and subsequent reduction of the levels of both methionine and SAMe (Barak et al. 1987; Finkelstein 1974; Trimble et al. 1993). Additionally, ethanol appears to enhance the loss of methyl groups, which in turn disrupts subsequent SAMe-dependent transmethylation reactions (Schalinske and Nieman 2005).

Rodent Models of Prenatal Ethanol Exposure

The teratogenic effects of prenatal alcohol exposure have been examined in rodent models for several decades. Studies have shown that in utero exposure to alcohol in these animals results in a wide range of anomalies, including growth retardation, CNS malformations, mental disability, and distinct craniofacial dysmorphology (Anthony et al. 2010; Boehm et al. 1997; Boggan et al. 1979; Bond and Di Giusto 1977; Klein de Licona et al. 2009; McGivern 1989; Parnell et al. 2009).

The FASD-like phenotypes observed in these rodent models have been associated with alterations in global gene expression, particularly in the developing brain (Hard et al. 2005; Hashimoto-

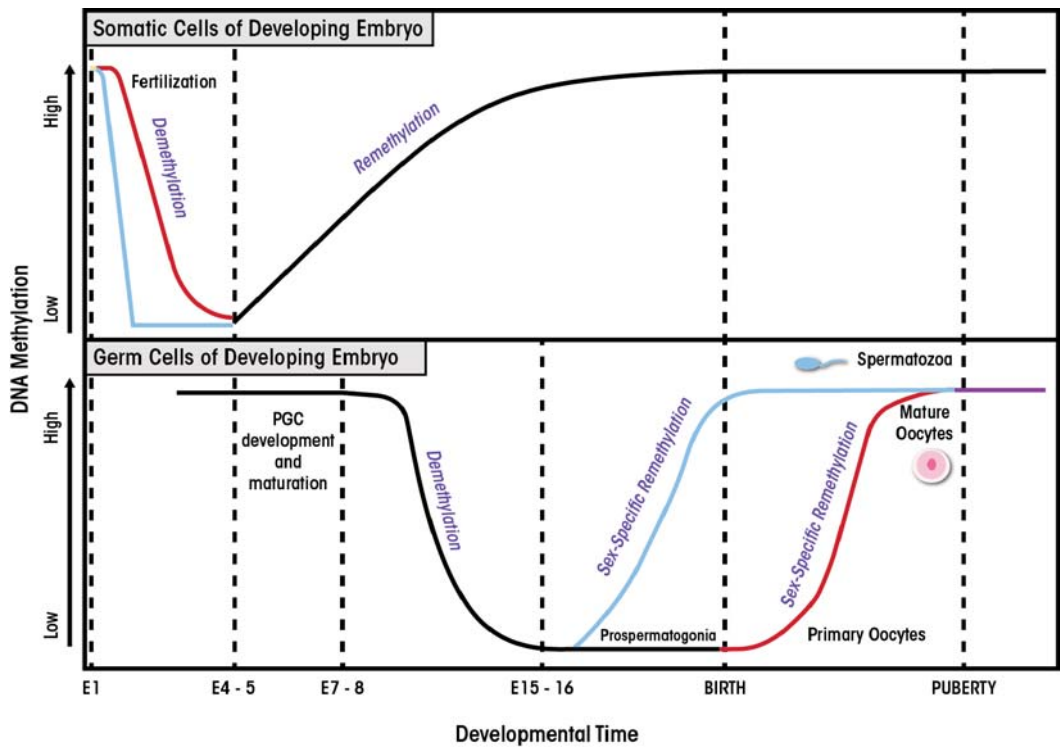

Figure 1 Reprogramming in mammalian development. Two waves of epigenetic reprogramming occur during embryo development. The first phase of reprogramming occurs in the normal body cells (i.e., somatic cells) of the developing embryo. In mice, following fertilization, the embryo undergoes genome-wide demethylation that is completed by embryonic day 5 (E5). The paternal genome (blue line) undergoes rapid, active demethylation, whereas in the maternal genome (pink line), demethylation occurs via a passive process. Remethylation of the embryonic genome begins at day E5 and is completed prior to birth. The second wave of epigenetic reprogramming occurs in the germ cells of the developing embryo, which will ultimately give rise to gametes that contain sex-specific epigenetic signatures. The primordial germ cells (PGCs) of the developing embryo contain the methylation signatures of the parental genomes. At approximately E7–8, the PGCs undergo rapid demethylation that is complete by E15–16. Following this, sex-specific methylation is re-established. In the male germline, reprogramming is complete at birth (blue line), whereas in females, reprogramming continues until puberty (pink line).

SOURCE: Adapted from Reik et al. 2001; Smallwood and Kelsey 2012.

Torii et al. 2011, 2011; Kleiber et al. 2012). This association, in conjunction with the vital role that epigenetic mechanisms play in controlling gene expression, suggests that normal epigenetic regulation by DNA methylation, histone modifications, and ncRNAs is disrupted as a result of ethanol insult.

Prenatal Ethanol Exposure and DNA Methylation

A direct link exists between ethanol exposure and aberrations in DNA methylation. For example, in a mouse model evaluating the effects of in utero ethanol exposure from days 9 to 11 of gestation, this acute ethanol administration resulted in lower-than-normal methylation throughout the genome (i.e., in global hypomethylation) of fetal DNA (Garro 1991). Furthermore, the ethanol-exposed fetuses displayed significantly reduced levels of DNA methylase activity. Ethanol-induced reductions in DNA methylation affect not only the fetus but also the placenta in pregnant mice exposed to alcohol (Haycock and Ramsay 2009). More recently, researchers evaluated the effect of prenatal alcohol exposure on DNA methylation of five imprinted genes in male offspring; these analyses detected a decrease in DNA methylation at a single locus in the *H19* imprinting control region in the sperm of these males (Stouder et al. 2011). Finally, in utero ethanol exposure in mice hinders the acquisition of DNA methylation in a brain region called the dentate gyrus, which is associated with developmental retardation (Chen et al. 2013).

Other analyses have looked at methylation patterns of specific genes rather than global DNA methylation. For example, a gene called *Agouti* has been used extensively as a model to study the effects of environmental (i.e., dietary) exposures on DNA methylation. The murine *Agouti* (*A*) locus regulates the animals' coat color; animals that carry two copies of the common variant, referred to as the wild-type allele, (i.e., *a/a* mice) display a pseudoagouti (i.e., brown) coat. A gene variant called A^{vy} is a dominant mutation that is caused by the insertion of a DNA sequence known as an intracisternal A-particle (IAP) retrotransposon in front of (i.e., upstream of) the *Agouti* gene. Animals that carry one mutant and one wild-type gene copy (i.e., heterozygous A^{vy}*/a mice*) display a variety of coat colors, ranging from yellow to mottled and brown, even though they are genetically identical. A^{vy} expression is strongly correlated to the DNA methylation profile of the inserted IAP. If the IAP shows hypomethylation, the *Agouti* gene is constantly expressed (i.e., shows constitutive ectopic *Agouti* expression) and the animals have a yellow coat. Conversely, hypermethylation correlates with promoter silencing and a pseudoagouti coat (Dolinoy et al. 2010). Kaminen-Ahola and colleagues (2010) investigated the effect of gestational ethanol exposure in A^{vy} heterozygous mice, demonstrating that ethanol exposure increased the proportion of pseudoagouti-colored offspring. This change in the proportion of coat colors was linked to transcriptional silencing of the mutant allele, which in turn correlated with hypermethylation of the A^{vy} locus. This study highlights the ability of prenatal alcohol exposure to alter the fetal epigenotype (albeit only at a specific locus) and, consequently, the adult phenotype.

In addition to the aberrant expression at the *Agouti* locus in the A^{vy} heterozygous mice, Kaminen-Ahola and colleagues (2010) noted altered gene expression profiles in the livers of their ethanol-exposed wild-type (*a/a*) siblings, as well as growth restriction and certain craniofacial dysmorphologies that are reminiscent of human FAS symptoms. Together, the findings that ethanol exposure can alter DNA methylation at the *Agouti* locus and elicit an associated phenotype (i.e., altered coat color), suggests that other epigenetic targets and associated gene expression also may be disrupted and may be responsible for the occurrence of a phenotype that corresponds to FAS in humans.

Similar studies have demonstrated the association of ethanol exposure with changes in DNA methylation and concurrent alterations in the expression of other genes. Downing and colleagues (2010) found that in utero ethanol exposure resulted in reduced methylation in the embryo at the *Igf2* locus, which encodes insulin-like growth factor 2, with a concomitant change in *Igf2* gene expression. These changes in gene expression were accompanied by skeletal malformations similar to those observed in FAS patients. In other studies, alcohol exposure resulted in neural tube defects in conjunction with genome-wide bidirectional methylation changes (i.e., occurrence of both hypo- and hypermethylation) (Liu et al. 2009). These altered methylation profiles were associated with significant changes in the expression of several genes associated with multiple functions, including chromatin remodeling, neuronal morphogenesis, synaptic plasticity, and neuronal development.

Together, these findings provide compelling evidence for alcohol-induced alterations of DNA methylation patterns in exposed fetuses that elicit a phenotype that is at least in part similar to that observed in FASD.

Prenatal Ethanol Exposure and Histone Modifications

Rodent models of alcoholism and in utero exposure to ethanol, as well as studies using cultured cells (i.e., in vitro experiments) have provided significant insights into the effects of alcohol on protein modifications, particularly to histones. Excess alcohol intake can exert its effect on protein modifications either directly or indirectly by disrupting the epigenetic machinery.

As with DNA methylation, some of these mechanisms involve folate, which as mentioned earlier serves as methyl group donor for histone methylation. Folate deficiency is a common clinical sign of chronic alcohol abuse and has been implicated in the development of alcoholism-related complications, such as alcoholic liver disease (Eichner et al. 1971). These deficiencies have been associated with significant alterations

in histone modifications, particularly at lysine residues (Esteller 2008; Kim and Shukla 2005; Park et al. 2003; Shukla et al. 2008). Altered histone modification, in turn, is associated with altered gene expression (Pal-Bhadra et al. 2007).

In in vitro studies using cultured rat liver cells (i.e., hepatocytes), ethanol exposure has been associated with bidirectional changes in histone methylation, including increased methylation at lysine 4 of histone H3 (i.e., increased H3K4me2) and decreased methylation at lysine 9 of histone H3 (i.e., decreased H3K9me2) (Pal-Bhadra et al. 2007). In addition, ethanol exposure led to selective acetylation of H3K9 (Park et al. 2003). These findings have been supported by in vivo models that have demonstrated increased H3K9 acetylation in the liver, lung, and spleen of adult rats acutely exposed to alcohol (Kim and Shukla 2006). Chronic alcohol exposure in adult rats also has been associated with increases in histone H3 and H4 acetylation in the amygdala of the brain that subsequently led to changes in the expression of the gene encoding a signaling molecule known as neuropeptide Y (Pandey et al. 2008). This increase in acetylation may result either from a decrease in the activity of the enzyme that removes acetyl groups (i.e., histone deacetylase) or an increase in the activity of the enzyme that adds acetyl groups (i.e., histone acetylase). Finally, in utero models have revealed that embryos exposed to acute levels of alcohol at mid-gestation showed elevated H3K9/18 acetylation as well as increased programmed cell death, referred to as apoptosis, of the fetal lung (Wang et al. 2010).

Zhong and colleagues (2010) investigated the effects of high and low levels of alcohol exposure on H3 acetylation and subsequent expression of genes related to heart development (i.e., *GATA4, Mef2c*, and *Tbx5*) in cardiac progenitor cells. Results indicated that low levels of alcohol increased H3 acetylation but did not significantly change the expression of the heart-development–related genes. In contrast, high levels of alcohol induced both H3 acetylation and significant gene-expression changes. These findings suggest that alterations to histone modifications are a potential mechanism for alcohol-induced cardiac disease (Zhong et al. 2010). An additional study by Guo and colleagues (2011) assessed the effects of alcohol on histone modifications in the cerebellum. The investigators found that perinatal alcohol exposure decreased the expression and function of one type of histone acetyl transferase called CREB binding protein (CBP). Altered CBP function resulted in decreased lysine acetylation on histones H3 and H4 within the cerebellum, which may contribute to the motor-activity deficits observed in FAS/FASD patients.

Another epigenetic mechanism by which alcohol could exert an effect on the epigenome is though the action of microRNAs (miRNAs).

More recently, researchers investigated the effects of alcohol exposure on certain fetal neuronal stem cells (i.e., fetal cerebral cortical neuroepithelial stem cells) and associated gene expression. These analyses found that ethanol exposure led to significant reductions in the levels of H3K4me3 (which activates gene expression) and H3K27me3 (which represses gene expression) (Veazey et al. 2013). Despite the reduction in expression-activating H3K4me3 levels, both increased and decreased transcription was observed in the genes investigated. Furthermore, loss of the repressive methylation mark, H3K27me3, did not result in altered transcription levels.

Altered protein modifications in response to alcohol exposure also may involve proteins other than histones that contribute to other manifestations of FAS, including proteins involved in insulin signaling. People with FAS often exhibit an underdeveloped cerebellum (i.e., cerebellar hypoplasia) that is associated with impaired insulin-stimulated survival signaling. This impaired signaling is mediated by the body's inability to properly respond to insulin (i.e., insulin resistance) (Soscia et al. 2006). It has been posited that chronic in utero ethanol exposure produces both insulin resistance in the CNS and oxidative stress, which is thought to play a major role in alcohol-related neurobehavioral teratogenesis (de la Monte and Wands 2010). In an in vivo model, adult rats prenatally exposed to alcohol exhibited reduced insulin signaling and increased expression of genes that regulate insulin (i.e., genes encoding proteins called TRB3 and PTEN) in the liver (Yao and Nyomba 2008). The analyses further suggested that the observed hepatic insulin resistance induced by alcohol exposure was associated with reduced acetylation of the TRB3 and PTEN proteins.

Taken together these findings suggest that alcohol-induced protein, and particularly histone, alterations continue to provide alternative or additional layers of complexity to an epigenetic etiology for FASD.

Prenatal Ethanol Exposure and ncRNA Dysregulation

Another epigenetic mechanism by which alcohol could exert an effect on the epigenome is though the action of microRNAs (miRNAs). These small ncRNAs play a critical role in several key biological processes, especially during in utero development, including cell-cycle regulation, differentiation, and organ formation (i.e., organogenesis). Individual miRNAs can affect many target genes, silencing their expression either by preventing translation of the intermediate molecules (i.e., messenger RNAs [mRNAs]) that are generated during transcription or by causing mRNA cleavage. Experimental evidence indicates that the expression of miRNAs is altered following exposure

to alcohol during development, and this may be one of the mechanisms underlying alcohol-related teratogenesis (Sathyan et al. 2007; Wang et al. 2009).

miRNAs have been implicated in the development of brain damage in response to prenatal alcohol exposure. Miranda and colleagues (2010) have hypothesized that ethanol causes brain damage during development by promoting the cell cycle of neural stem cells. This would accelerate the maturation of these progenitor cells and result in their premature depletion. This hypothesis is compatible with the observation that when clusters of neural stem cells (i.e., neurospheres) are grown in culture, differentiating neuroblasts from these clusters show increased migration and depletion of stem cells when they are exposed to ethanol compared with their unexposed counterparts. This observed behavior suggests the involvement of a large network of genes controlling complex biological outcomes. In order to examine the trigger for this behavior, researchers examined miRNA expression levels in alcohol-exposed and nonexposed neural stem cells. A preliminary screen of miRNAs in neural stem cells identified four miRNAs (i.e., miR9, miR21, miR135 and miR355) that were suppressed in the presence of ethanol exposure (Sathyan et al. 2007). These miRNAs were found to act both antagonistically and synergistically, both reducing and promoting apoptosis. Normally, these miRNAs favor normal development by balancing cell survival and cell proliferation. Following alcohol exposure, however, the reduction in their levels leads to an imbalance with detrimental effects.

In another study (Wang et al. 2009), pregnant mice were exposed to ethanol from day 6 to day 15 of gestation, and fetal brain tissue was examined for differential miRNA expression. Under these conditions, seven miRNAs were upregulated and eight were downregulated in response to ethanol exposure, with miR10a and miR10b showing the highest level of overexpression. It is biologically plausible that overexpression of these two miRNAs can disrupt developmental processes because they are thought to regulate expression of a group of genes called the *Hoxb* gene family (Wang et al. 2009). This group of genes is involved in the regulation and establishment of body patterning during embryonic development. Interestingly, there was no overlap in the miRNAs between this study and those identified in the study by Sathyan and colleagues (2007), suggesting that different models for alcohol exposure as well as the investigation of different tissues and different developmental time periods of exposure may have varying impacts on diverse miRNA targets.

Taken together, the preliminary studies suggest that miRNA plays a crucial role in normal development and that this process can be disrupted by alcohol exposure during critical periods, especially during neurogenesis.

Role of Preconception Alcohol Exposure in FASD

Although studies of FASD etiology predominantly have focused on maternal exposure during pregnancy, evidence also exists in support of contributions of paternal exposure. For example, FAS-like effects have been observed in children of alcoholic fathers even in the absence of gestational alcohol exposure, suggesting the possibility that preconception alcohol exposure may affect offspring development (Abel and Tan 1988; Lemoine et al. 1968). Studies conducted in rodents 100 years ago have supported these findings (Stockard 1913; Stockard and Papanicolaou 1916), and more recent analyses also reported that paternal preconception alcohol exposure was associated with neurobehavioral abnormalities, low birth weights, congenital malformations, and growth retardation in offspring (Friedler 1996; Jamerson et al. 2004). Additional studies have implicated a role for altered sperm DNA methylation in paternally-mediated effects of preconception ethanol exposure on offspring development (Knezovich and Ramsay 2012).

Transgenerational Transmission of the Effects of Alcohol Exposure

Altered epigenetic modifications (i.e., epimutations) may also be passed on from one generation to the next. There are two modes in which such a transmission of epimutations can occur (Skinner 2008):

- Multigenerational inheritance, in which several generations are affected because they all are exposed to the same factor (e.g., alcohol) and thus are prone to the same modifications; and

- Transgenerational inheritance, which involves a reprogramming event in the germline in response to a specific factor (e.g., alcohol exposure), resulting in an altered epigenome that would be inherited by future generations even if they are not themselves exposed to the same factor.

It was previously believed that transgenerational epigenetic inheritance would be unlikely because, as mentioned previously, epigenetic reprogramming occurs in the germline. However, increasing evidence indicates that transgenerational epigenetic inheritance does indeed happen (Anway et al. 2005; Crepin et al. 2012; Stouder and Paoloni-Giacobino 2010). Most of the work conducted thus far in this area has focused on the effects of agents that can interfere with the body's normal hormone systems (e.g., vinclozolin, which affects sex hormone levels and has been shown to have transgenerational effects). The potential transgenerational effects of alcohol and their role in the etiology and perpetuation of FAS/FASD symptoms in affected individuals and their progeny, however, still need to be determined.

Conclusions

Evidence is rapidly accumulating in support of an epigenetic etiology in the development of FASD (figure 2). All three types of epigenetic modulators—DNA methylation, histone modifications and regulation by ncRNAs—are perturbed by ethanol exposure. These ethanol-related changes can affect gene expression of critical developmental genes and pathways, impacting cell proliferation and differentiation.

The phenotypic consequences of in utero ethanol exposure are significantly correlated with the molecular consequences of ethanol's effects on epigenetic regulatory mechanisms. A complex picture of locus-specific and cell-type–restricted effects is emerging. In particular, many studies have focused on ethanol's effects on mechanisms that regulate neurogenesis, leading to the most devastating consequences of alcohol exposure during development. The range of effects appears to be significantly influenced by the timing and level of exposure, leading to a wide range of outcomes and combinations of phenotypic indicators.

In mouse models, ethanol exposure can be carefully controlled and other environmental parameters, such as diet and stress, can be kept constant. This allows for careful investigation of the effects of alcohol exposure on epigenetic regulatory mechanism and their association with FAS-like symptoms. Drinking patterns in pregnant women, in contrast, are seldom accurately documented and often occur throughout gestation, which, not surprisingly, leads to a vast array of phenotypes now recognized under the banner of FASD. Thus, discerning the role of epigenetic mechanisms in these processes will be much more challenging. ■

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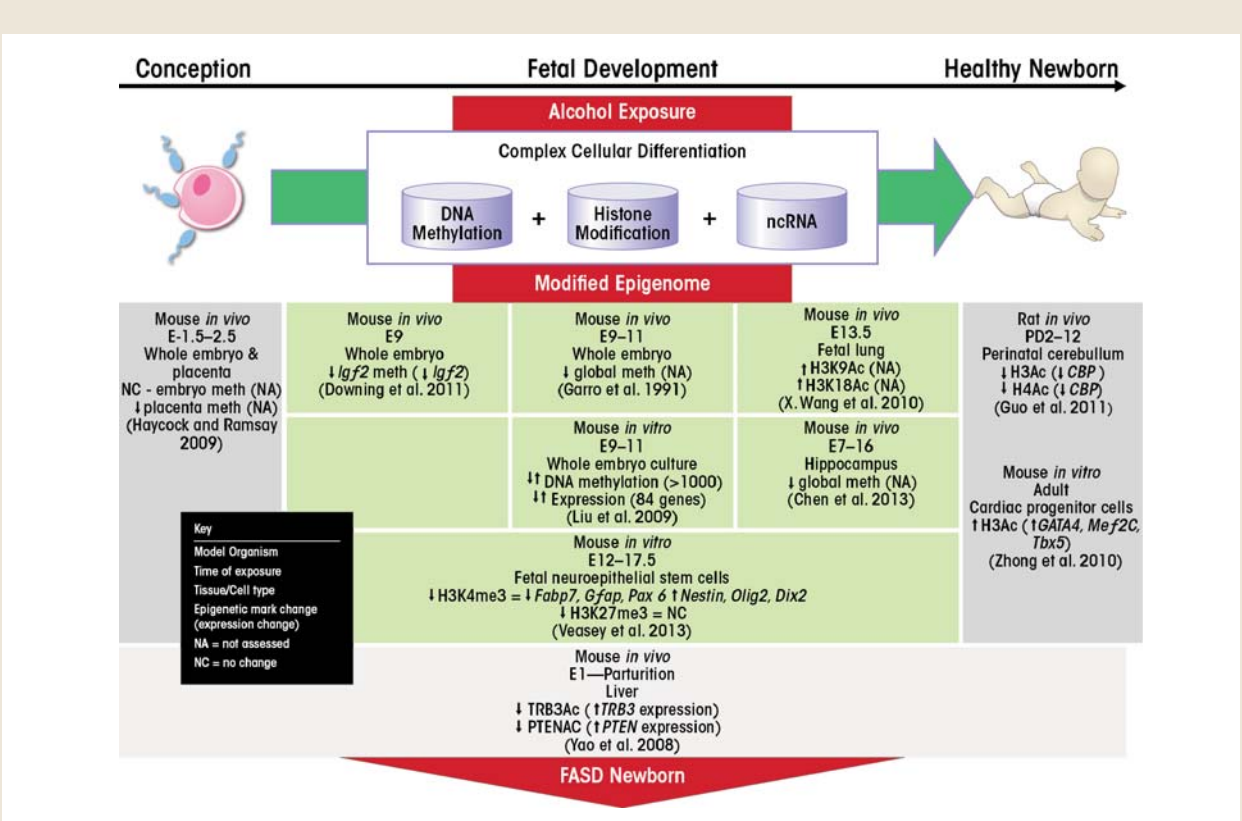

Figure 2 Epigenetic contributions to FASD. Following conception, a complex orchestration of epigenetic mechanisms ensures normal cellular differentiation and embryonic development (green horizontal arrow). These mechanisms include DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) to modulate gene expression in a specified temporal and spatial manner. Alcohol exposure in utero (red downward arrow) has been shown to alter these epigenetic modulators, which may consequently dysregulate gene expression patterns as indicated by the study findings listed and affect normal embryonic development and phenotype outcome. By these mechanisms, alcohol-induced epigenetic aberrations may contribute to the etiology of fetal alcohol spectrum disorders (FASD).

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Dysregulation of microRNA Expression and Function Contributes to the Etiology of Fetal Alcohol Spectrum Disorders

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MicroRNAs (miRNAs) are members of a large class of non–protein-coding RNA (ncRNA) molecules that represent a significant, but until recently unappreciated, layer of cellular regulation. Assessment of the generation and function of miRNAs suggests that these ncRNAs are vulnerable to interference from genetic, epigenetic, and environmental factors. A small but rapidly expanding body of studies using a variety of animal- and cell culture–based experimental models also has shown that miRNAs are important targets of alcohol during fetal development and that their dysregulation likely plays a significant role in the etiology of fetal alcohol spectrum disorders (FASD). Accordingly, an analysis of the regulation and function of these miRNAs may yield important clues to the management of FASD. **Key words: fetal alcohol spectrum disorders; fetal development; microRNAs; cellular regulation; genetic factors; epigenetic factors; environmental factors; animal models; cell culture studies**

MicroRNAs (miRNAs) are members of a vast, evolutionarily ancient, but poorly understood class of regulatory RNA molecules, termed non–protein-coding RNAs (ncRNAs). This means that in contrast to RNA molecules generated during gene expression (i.e., messenger RNA [mRNA] molecules), they are not used as templates for the synthesis of proteins. ncRNAs are encoded within the genomes of both eukaryotic and prokaryotic organisms and represent a novel layer of cell regulation and function that rivals the diversity and function of protein-coding mRNAs (for review, see Mattick 2007).

In recent years, researchers have investigated whether, and how, miRNAs interact with beverage alcohol (i.e., ethanol) and/or mediate its effects. Initial studies (Sathyan et al. 2007) explored the ethanol–miRNA interactions in fetal neural stem cells. Since then, increasing evidence has indicated that miRNAs play a role in the etiology of alcoholism (Pietrzykowski et al. 2008) and potentially alcohol withdrawal (Guo et al. 2011), as well as in ethanol's effects on brain development (Guo et al. 2011; Tal et al. 2012; Wang et al. 2009), brain damage associated with adult alcoholism (Lewohl et al. 2011), and liver damage (i.e., hepatotoxicity) (Dolganiuc et al. 2009; Tang et al. 2008). Other drugs of abuse such as nicotine are also known to influence miRNA expression (Huang 2009); furthermore, ethanol and nicotine collaborate to regulate the expression of miRNAs in neural tissues (Balaraman et al. 2012). These data collectively suggest that miRNAs are an important, but as yet poorly understood, component of alcoholism and ethanol-associated toxicology and damage to the developing fetus (i.e., teratology). This review specifically focuses on the association between miRNAs and the developmental effects of ethanol exposure, examining both the current data and future potential for research in this field of ncRNA biology to promote a coherent understanding of teratology associated with alcohol exposure.

Fetal Alcohol Spectrum Disorders

Maternal alcohol consumption during pregnancy can lead to a constellation of brain, face, cardiovascular, and skeletal defects of varying severity that collectively have been termed fetal alcohol spectrum disorders (FASD). At the extreme end of the spectrum of severity is fetal alcohol syndrome (FAS) (Clarren 1986), which is characterized by craniofacial abnormalities (e.g., small openings of the eyes, thin upper lip, flattened area above the upper lip),

motor dysfunction, impaired coordination of muscle movements (i.e., ataxia), behavioral disturbances, and cognitive deficits as well as growth retardation (Jones et al. 1973). According to the Centers for Disease Control and Prevention, the incidence of FAS is 1 to 3 per 1,000 live births, and these rates increase to 10 to 15 per 1,000 in at-risk groups, such as the foster care population (May and Gossage 2001). More recent estimates suggest that the prevalence of FASD in school-aged children in the United States is between 2 and 5 percent (May et al. 2009). FASD imposes significant socioeconomic costs on families and society. The lifetime cost of caring for a child with FASD was estimated at about $2 million, and the total annual cost of FASD in the United States was estimated at $4 billion in 2004 (Lupton et al. 2004); these costs may be significantly higher today.

Although the facial characteristics seen in patients with FASD are the most obvious signs of fetal alcohol exposure, the most devastating consequences of prenatal alcohol exposure are brain defects that result in cognitive, affective, and motor deficits (Sampson et al. 2000). Therefore, understanding the diverse effects of alcohol on the developing brain during pregnancy may provide researchers with the key to developing therapies for managing both fetal and adult effects of alcohol exposure during pregnancy. This review focuses on an emerging body of data from animal and cell-culture studies that implicates miRNA dysregulation in the etiology of FASD.

Focus on miRNAs

miRNAs are a class of ncRNAs that posttranscriptionally regulate the expression of protein-coding genes. When protein-coding genes are expressed (i.e., the encoded protein is produced), first an mRNA copy of the corresponding DNA sequence is generated in a process called transcription. This mRNA molecule consists of three parts: a noncoding start region (i.e., the 5'-end), the sequence actually containing the information for the encoded protein (i.e., the open reading frame), and a noncoding tail region (i.e., the 3'-end). miRNAs mainly act by binding to the 3'-untranslated region of their mRNA targets (Ambros et al. 2003; Bartel 2004; Ghildiyal and Zamore 2009), although that is not the only function attributable to these molecules. Many microRNAs are evolutionally conserved across species. They initially were discovered in the roundworm *Caenorhabditis elegans* (Lee et al. 1993), but since then they also have been found in plants, invertebrates, mammals, and humans (Bartel 2009). miRNAs play crucial roles in development, stem-cell self-renewal, programmed cell death (i.e., apoptosis), and cell-cycle regulation but also feature prominently in human disease, including cancers and neurodegenerative and metabolic diseases (Ambros 2004; Bartel 2004). miRNAs are abundant in the central nervous system (CNS) (Krichevsky et al. 2003; Vreugdenhil 2010), and brain miRNAs are crucial for regulating nerve cell generation (i.e., neurogenesis); neuronal degeneration; and maintaining normal neuronal functions associated with memory formation, neuronal differentiation, and synaptic plasticity (Li 2010; Schratt 2009; Smalheiser 2009).

miRNA Biogenesis

miRNAs are encoded within the genome either as independent genes or in gene clusters; however, they also can be encoded within introns[1] of protein-coding genes, or even within introns and exons of another type of ncRNA called long intergenic non-(protein)-coding RNAs (lincRNAs). The generation of mature miRNAs from these coding sequences is a multistep process, as follows (see figure 1A):

- A normal transcription process, which is mediated by an enzyme called RNA-polymerase II, generates a longer primary transcript termed pri-miRNA. Like mRNA, the pri-miRNA transcripts can have certain modifications at their ends (i.e., a "cap" at the 5'-end and multiple adenosine units [i.e., a poly-A tail] at the 3'-end) and can be spliced (Cai et al. 2004). Furthermore, the pri-miRNAs typically are folded into a double-stranded, hairpin–loop structure several hundred base pairs in length.

- Most pri-miRNA transcripts are processed within the nucleus by a protein complex called the DiGeorge syndrome critical region-8 (Drosha/DGCR8) "microprocessor" complex to generate stem-loop structures termed pre-miRNAs that are approximately 70 nucleotides in length (Han et al. 2006; Lee et al. 2003).

- The pre-miRNAs are moved from the nucleus to the cytoplasm by a chaperone protein called exportin-5 (Bohnsack et al. 2004).

- Within the cytoplasm, a protein complex known as Dicer enzyme further processes pre-miRNAs into mature double-stranded miRNA molecules (Hutvagner et al. 2001; Zhang et al. 2002). This process, and thus miRNA formation in general, is crucial for embryonic development because mutations in the Dicer proteins, which are exclusively part of the miRNA processing machinery, cause death of the embryo (Bernstein et al. 2003).

- Once the Dicer complex is cut off to release the mature miRNA, one strand of the double-stranded molecule, termed the guide strand miRNA, preferentially attaches to another protein complex called

[1] Genes encoding specific proteins typically comprise not only the DNA sequences that contain the building instructions for the resulting protein, but also noncoding sequences (i.e., introns). These introns are interspersed with the coding sequences (i.e., exons). During transcription, first an RNA copy of the entire gene, including introns and exons, is generated. This transcript is then processed by cutting out the intron sequences, to generate the final mRNA molecule. This process is known as splicing.

RNA-induced silencing complex (RISC). This results in a microribonucleoprotein (miRNP) complex that can either destabilize mRNA transcripts or repress the next step of gene expression in protein-coding genes (i.e., translation) (Mourelatos et al. 2002; Williams 2008). The second, complementary strand, known as passenger strand or miRNA* (see figure 2) has been thought to be quickly degraded (Matranga et al. 2005). However, as discussed later in this article, recent studies indicate that passenger-strand miRNAs can be retained by cells and exhibit independent biological functions.

- Finally, the mature miRNA can be degraded by an enzyme called 5'-3' exoribonuclease (XRN2) (Bail et al. 2010).

Role of miRNAs in Ethanol's Teratologic Effects

In 2007, Sathyan and colleagues (2007) showed for the first time that miRNAs could mediate the effects of ethanol or indeed other teratogens. Using isolated tissue from the nervous system (i.e., neuroepithelium) of second-trimester fetuses, the investigators demonstrated that ethanol suppressed the expression of four miRNAs—miR-9, miR-21, miR-153, and miR-335—in fetal neural stem cells (NSCs) and neural progenitor cells (NPCs). The simultaneous suppression of miR-21 and miR-335 accounted for earlier observations (Prock and Miranda 2007; Santillano et al. 2005) that ethanol-exposed NSCs/NPCs are resistant to apoptosis, whereas the suppression of miR-335 explained the increase in NSC/NPC proliferation. Three of the four suppressed miRNAs target the mRNAs for two proteins called Jagged-1 and ELAVL2/HuB;[2] accordingly, by suppressing the miRNAs, ethanol induced the expression of both target mRNAs. ELAVL2/HuB overexpression promotes neuronal differentiation (Akamatsu et al. 1999), and Jagged-1–induced proliferation establishes neuronal identity (Salero and Hatten 2007). These data collectively suggest that by interfering with miRNA function, ethanol may deplete the fetal pool of NSCs/NPCs and promote premature neuronal differentiation. More recently, Tal and colleagues (2012), using a zebrafish model, also showed that ethanol exposure during embryonic development suppressed the expression of miR-9 and miR-153. Importantly, these investigators demonstrated both behavioral and anatomical consequences of miRNA depletion. In particular, miR-153 depletion resulted in significantly increased locomotor activity in juvenile zebrafish, reminiscent of increased hyperactivity observed in children with FASD.

Other developmental ethanol exposure models also have indicated that ethanol alters the expression of several miRNAs. For example, Wang and colleagues (2009) showed that ethanol exposure during a period bracketing the end of the first trimester to the middle of the second trimester resulted in altered miRNA expression in brain tissue sampled near the end of the second trimester. In that study, ethanol induced a significant increase in the expression of two miRNAs (i.e., miR-10a and miR-10b), resulting in down-regulated expression of a protein called Hoxa1 in fetal brains. Other analyses had indicated that loss of Hoxa1 function (e.g., from familial Hoxa1 mutations) is associated with a variety of cranial defects and mental retardation (Bosley et al. 2007). This suggests that by suppressing translation of Hoxa1 and related genes, ethanol-mediated induction of miR10a/b may lead to similar defects.

Although the miRNAs identified by Wang and colleagues (2009) do not overlap with those identified by Sathyan and colleagues (2007) in NSCs/NPCs, miR-10a/b upregulation may have similar consequences for premature NSC differentiation. For example, miR-10a/b promotes the differentiation of cells from a type of nerve cell tumor (i.e., neuroblastoma cells) by suppressing translation of a protein called nuclear receptor corepressor-2 (NCOR2) (Foley et al. 2011). This effect is similar to the induction of Elavl2/HuB and Jagged-1.

Finally, Guo and colleagues (2011) assessed the effects of chronic intermittent ethanol exposure on cultured neuronal cells obtained from mouse cerebral cortex at gestational day 15, which is equivalent to the middle of the second trimester. The investigators found that ethanol induced several miRNAs in these cells. Interestingly, a prolonged period of withdrawal following the ethanol exposure resulted in a more than fourfold increase in the number of significantly regulated miRNAs, suggesting that withdrawal itself also may have a significant damaging effect on neuronal maturation in the developing fetal brain. Although these data were obtained from a cell-culture model, the implications of maternal binge drinking–withdrawal cycles on fetal miRNAs and their control over neural differentiation need further investigation.

Effects of Coexposure to Ethanol and Other Drugs on miRNA Levels

Pregnant women who abuse ethanol also are likely to coabuse other drugs, such as nicotine (Substance Abuse and Mental Health Administration 2009). These other drugs also can affect miRNA levels. For example, Huang and colleagues (2009) demonstrated that nicotine induced expression of miR140* in a developmental model using the rat PC12 cell line. These effects may enhance or oppose those of ethanol. Thus, a recent study showed that ethanol and nicotine behaved as functional antagonists—that is, miRNAs that were suppressed by ethanol in fetal NSCs/NPCs were induced by nicotine exposure (Balaraman et al. 2012). Moreover, nicotine prevented the ethanol-mediated decrease in these miRNAs; this effect was pharmacologically mediated

[2] Jagged-1 binds to (i.e., is a ligand of) Notch receptor and ELAVL2/HuB is a neuron-specific RNA-binding protein.

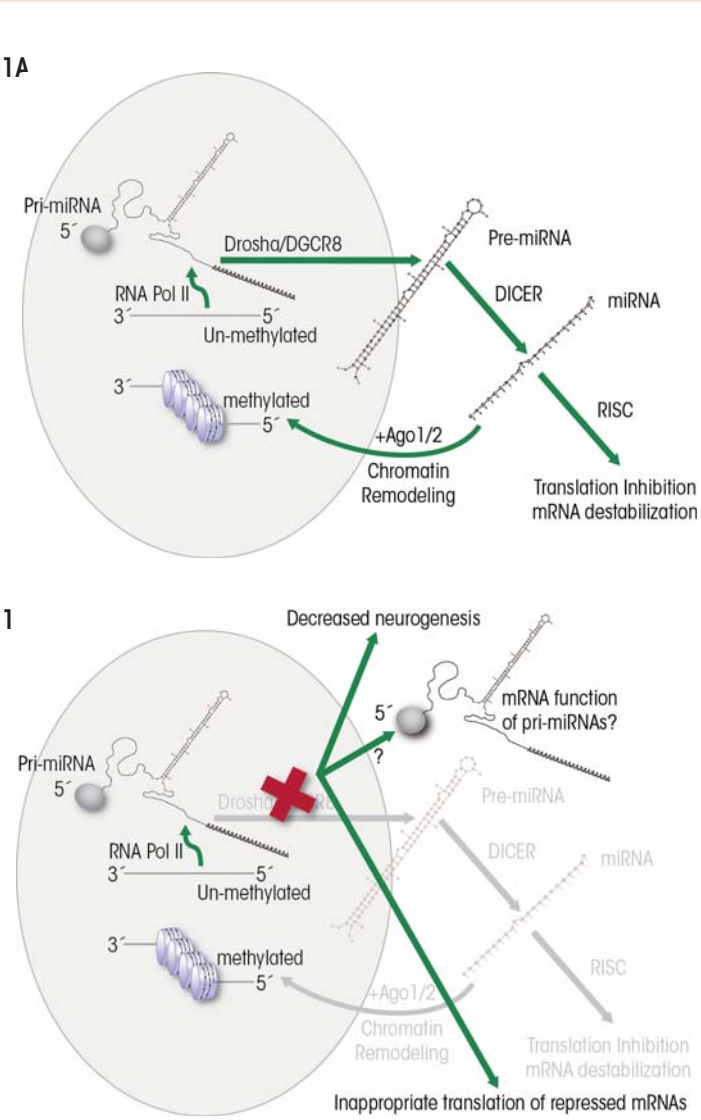

Figure 1 Models for standard (i.e., canonical) and disturbed (i.e., noncanonical) modes of miRNA biogenesis and function. **(A)** miRNAs often are generated (i.e., transcribed) from miRNA genes, as long mRNA-like transcripts, with a "cap" at the start (i.e., 5′-end) and several adenosine units at the end (i.e., 3′- polyA tail). The initial primary miRNA transcripts (pri-miRNAs) are processed to shorter, hairpin-shaped premature miRNAs (pre-miRNAs) by a protein complex called the DiGeorge syndrome critical region-8 (Drosha/DGCR8) complex. The pre-miRNAs then are transported to the cytoplasm for final processing into mature miRNAs by the Dicer complex. Mature miRNAs attain their function by being integrated into RNA-induced silencing complexes (RISC) that can degrade target mRNAs or silence translation. Processed miRNAs also can relocate to the nucleus to influence chromatin remodeling. **(B)** Disturbances in Drosha/DGCR8 processing (e.g., because of a mutation in the genes encoding these enzymes) may reveal alternate, mRNA-like functions of unprocessed pri-miRNAs and result in disrupted stem cell maturation.

by a certain type of nicotine receptor (i.e., the nicotinic acetylcholine receptor). There is little generalized evidence as yet that drugs of abuse interact at the level of miRNAs to regulate cell function. Nevertheless these findings suggest that such an interaction is a real possibility, and the consequences for the teratologic effects of the drugs are likely to be significant.

Teratogenic Implications of Altered miRNA Biogenesis, Cellular Localization, and Function

The data cited above show that ethanol alters the expression of several miRNAs at different developmental stages and that these alterations have consequences for fetal neural development and behavior. miRNA dysregulation is likely to influence teratogenesis by destabilizing the mRNAs of individual genes or gene networks. However, emerging evidence indicates that miRNA function also can be altered at several stages in the miRNA biogenesis pathway. Although to date such alterations are poorly understood, they may have important implications for teratology. The following represent four intriguing possibilities.

First, the presence of a 5' cap and a 3'-polyA tail indicates that primary miRNA transcripts may have characteristics and function like regular mRNAs, and indeed evidence has been found for such a role (Cai et al. 2004). Although the conditions that permit the appearance of mRNA-like functionality are unclear, it is likely that interference with Dicer/DGCR8, which is essential for miRNA processing, can lead to the emergence of alternate functionality associated with pri-miRNA transcripts (see figure 1B). In this context, it is interesting to note that disruption of the DGCR8 locus is associated with mental retardation and that DGCR8 deletion interferes with the maturation of embryonic stem cells, causing them to aberrantly retain their ability to differentiate into differ-

ent cell types (i.e., their pluripotency) while initiating differentiation (Wang et al. 2007). In this instance, the biology of stem cells seems to be intimately linked with the development of normal brain function.

Second, until recently, the complementary miRNA* strands (Hutvagner et al. 2001) were thought to be quickly degraded following Dicer cleavage of the double-stranded pre-miRNA molecule (Matranga et al. 2005). However, recent evidence (Ghildiyal et al. 2010; Okamura et al. 2009; Tyler et al. 2008) shows that these passenger strands also can be functional, acting on their own binding sites and regulating expression of their own sets of targets (figure 2). Thus, both strands of a pre-miRNA can be functional, each with a specific set of targets. The ratio of functional guide versus passenger strand miRNAs is regulated by an as-yet-unknown biology. Guo and colleagues (2011) identified several ethanol-sensitive miRNA* species that mainly were induced following ethanol exposure. Furthermore, other drugs of abuse, such as nicotine, also have been shown to induce the expression of a miRNA* (i.e., miR140*) (Huang and Li 2009). Alterations in the miRNA-to-miRNA*s ratio are likely to yield alternate biological outcomes that are particularly relevant to teratogenesis, as has been demonstrated in an analysis of the established ethanol-sensitive miRNA, miR-9. Tal and colleagues (2012) showed in their developmental zebrafish model that in addition to decreasing miR-9 (which now is also called miR-9-5p [www.mirbase.org, miRBAse Release 19]), ethanol produced a more modest decrease in the expression of miR-9* (now called miR-9-3p). The ratio of miR-9 to miR-9* is important for development and teratogenesis because these two miRNAs work together to regulate two molecules controlling neuronal differentiation. Thus, miR-9 levels influence the levels of a neuronal differentiation inhibitor called RE1 silencing transcription factor/neuron-restrictive silencer factor (REST), whereas miR-9* regulates its cofactor, coREST (Packer et al. 2008). Therefore, the simultaneous suppression of miR-9 and miR-9* may be expected to result in derepression of the REST/coREST complex and, consequently, inhibition of neuronal differentiation. On the other hand, preferential suppression of either miR-9 or miR-9* would be predicted to alter the ratio of REST to coREST, which has important and complex consequences for neural stem-cell renewal and altered lineage specification (Abrajano et al. 2009, 2010). Clearly, the involvement of passenger-strand miRNA biology in teratogenesis needs further investigation.

Third, in humans, about 6 percent of mature miRNAs undergo editing by the enzyme adenosine deaminase (Blow et al. 2006), resulting in alterations in either miRNA processing or miRNA efficiency (Kawahara et al. 2007; Yang et al. 2005). Furthermore, evidence suggests that edited miRNAs may exhibit different target specificity compared with their nonedited counterparts (Ekdahl et al. 2012). miRNA editing increases during brain development and may permit the emergence of new biological functions (e.g., a novel translational control of the development of nerve cell extensions [i.e., dendritogenesis]) (Ekdahl et al. 2012). These data collectively suggest that the role of miRNA editing in ethanol teratology warrants further exploration.

Finally, emerging evidence indicates that some mature miRNAs are transported back to the nucleus, where they mediate the formation of heterochromatin[3] (Gonzalez et al. 2008). This observation suggests that miRNAs can directly influence the epigenetic landscape (figure 1A). Ethanol also alters

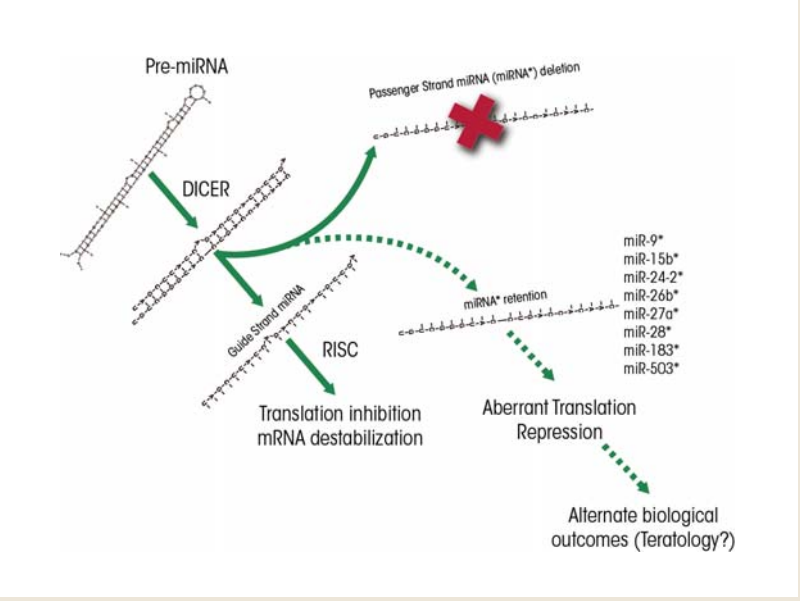

Figure 2 Model for the activity of the two strands of the processed pre-miRNA molecules (i.e., the guide strand [miRNA] and passenger strand [miRNA*]). Dicer processing of pre-miRNAs typically results in the formation of a guide stand miRNA that binds to the RNA-induced silencing complex (RISC). This guide strand can be derived from either the 3′- (termed –3p) or 5′- (termed –5p) end of the pre-miRNA. The complementary passenger strand typically is degraded. However, under various conditions, including ethanol exposure, miRNA* strands may be retained or otherwise differentially regulated, resulting the emergence of alternate biological end points.

SOURCE: Guo et al. 2012, Acer 2012, Tal et al. 2012.

the epigenetic landscape in differentiating fetal NSCs (Zhou et al. 2011), and the contributory role of nuclear miRNAs to this process is unknown. All of these modifications to miRNA biology represent novel and uninvestigated layers of regulatory processes that may have important consequences for cell and tissue differentiation and, consequently, teratogenesis.

Implications for the Management of FASD

Despite strong evidence that maternal alcohol consumption during pregnancy leads to harmful effects on the fetus, a significant number of women continue to report drinking even into the third trimester of pregnancy. Therefore, early detection and management of fetal alcohol exposure remains an urgent public health concern, as does the development of approaches to ameliorate or prevent ethanol's detrimental effects. The identification of miRNAs as ethanol targets presents one hope for the development of novel therapeutic programs. miRNAs have coevolved with their mRNA targets to orchestrate development. It is possible that miRNA-like drugs may be used to mitigate the effects of fetal ethanol exposure on the development of specific organs. The challenge will be to identify tissue-specific miRNAs that can be used to reprogram development. In this context, miRNAs such as miR-9 make intriguing therapeutic targets because they are fairly specific to neuronal cells (Leucht et al. 2008; Shibata et al. 2011; Smirnova et al. 2005). Evidence that ethanol-sensitive miRNAs also are sensitive to nicotine (Balaraman et al. 2012) suggests a promising and alternative, pharmacological approach to reprogramming fetal development following maternal ethanol exposure. Recent evidence suggests that pharmacologic approaches can indeed be used successfully in human populations, for example, to normalize cellular miRNA levels in neurological diseases such as multiple sclerosis (Waschbisch et al. 2011). Such an approach therefore may be similarly efficacious with FASD. Finally, Guo and colleagues (2011) have implicated DNA methylation as a mechanism for miRNA regulation, and Wang and colleagues (2009) demonstrated that folic acid administration could reverse ethanol's effects on miRNAs. These data suggest that nutritional supplementation programs also may be an effective means towards ameliorating the effects of miRNA dysregulation. Research into miRNA involvement in fetal alcohol teratology is in its infancy. However, this research has significant potential for both uncovering principles underlying alcohol's detrimental consequences and for developing novel strategies for the management of fetal alcohol effects. ■

[3] Heterochromatin is a tightly packed complex of DNA and proteins in the cell nucleus in which the DNA is not easily accessible to other proteins and therefore generally is not expressed. In contrast, a more lightly packed DNA–protein complex (i.e., euchromatin) is more easily accessible for transcription enzymes.

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