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Substance Abuse and Early Development

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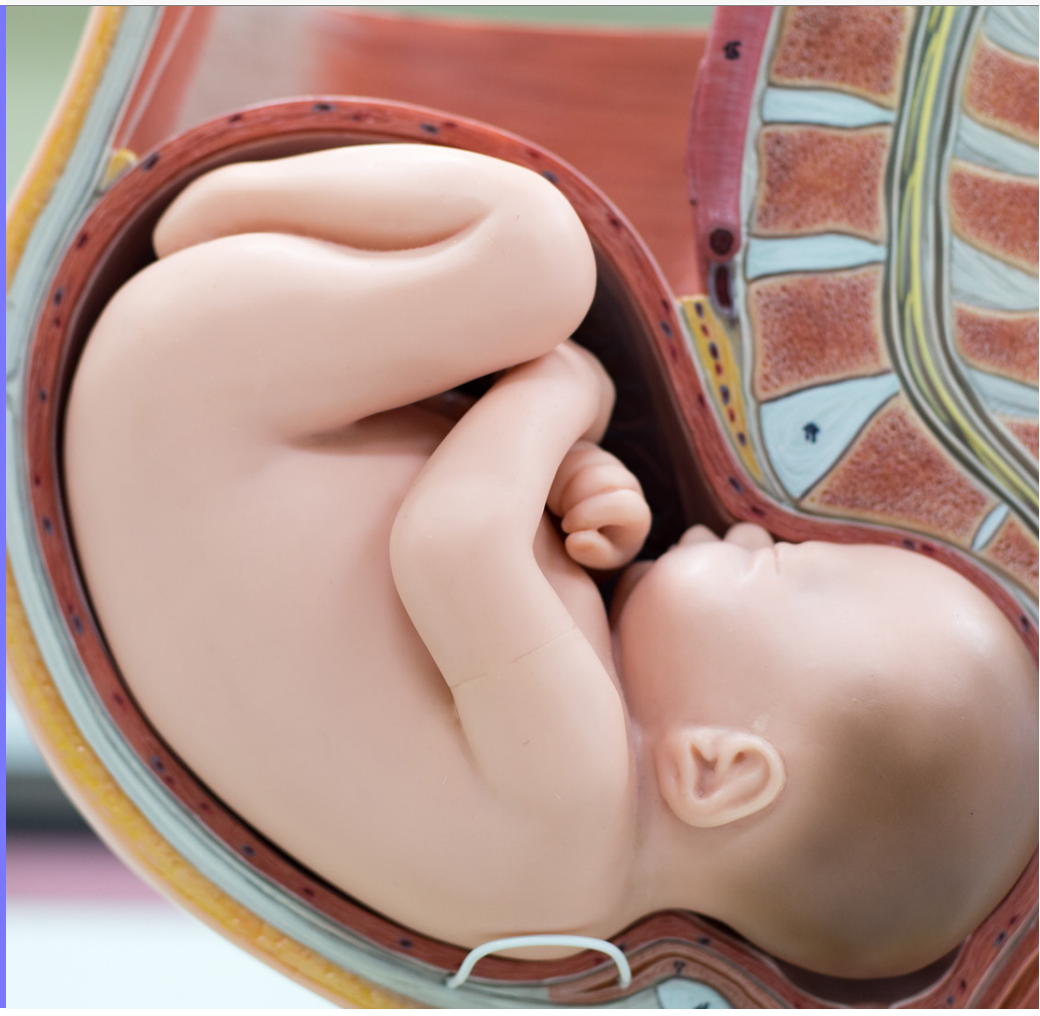

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Substance Abuse and Early Development

Substance abuse in the form of tobacco, alcohol, drug, or chemical misuse affects the entire family. Besides devastating consequences on relationship dynamics, substance abuse by prospective parents has an invisible target - the progeny. While consequences of some substance use (such as alcohol) on early and fetal development are well known, others are less studied. This Special Issue aims at describing recent advances in our understanding of how parental substance abuse affects early development of progeny.

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Editorial: Substance abuse and early development

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prenatal, drug abuse, narcotics, developmental trajectory, developmental toxicity

Editorial on the Special Issue
Substance abuse and early development

While misuse of psychoactive substances can be traced back to pre-historic times, their effects on invisible target—such as the developing fetus—only gained recognition within the last century [1–4]. Initial seminal findings in humans and laboratory rodents quickly grew from isolated observations to well-planned, multidisciplinary studies. Despite the involvement of numerous methodologies, large teams of researchers, and widely different experimental organisms, there has been an unusual consensus on the meaning of findings: principally that prenatal exposure to drugs of misuse has deleterious consequences for the developing fetus.

The mechanisms by which prenatal exposure to psychoactive substances alters developmental trajectories are variable, from epigenetic alterations to fine-tuning of blood supply to the brain [5, 6]. In some cases, such as during cannabis exposure, the impact is exacerbated by the vast presence and activity of the endocannabinoid system during early development (Fride, 2008; [7]). In others, such as with alcohol, efforts for elucidating potential mechanisms of alcohol-driven alterations and thus, identifying venues for novel therapeutic options are hindered by the chemical's simple structure and the abundant sensing sites it can bind to. Additional challenges are presented by the growing trend of substance co-use, such as in cases of simultaneous consumption of alcohol and marijuana [8] or opioid and stimulant products [9]. In these instances, in addition to the effects of individual drugs, their potential interactions must be considered.

The current Special Issue aims at describing recent advances in our understanding of how maternal and paternal substance abuse affects early development of progeny. Contributions into this Special Issue span from methodological advancements, into reviews of current standing in the field, original research report, and novel diagnostic tools. Specifically, Mbolle et al. take the reader on a journey into modern technology for high-resolution imaging of prenatal development. While this task is certainly streamlined in obstetrics clinics, major challenges remain at the bench, as current resolution capabilities are often below the thresholds needed for accurate visualization of fetal structures within small rodent species such as mice which are used for mechanistic studies. Development of deep-tissue high-resolution imaging and accompanying

computational reconstruction technology will serve many researchers who are trying to elucidate the underpinnings of prenatal drug exposure effects. Before such methodology could advance the field, a thorough analysis of existing knowledge is needed, so that gaps could be identified and pursued with precision targeting. To address this task, Mulligan and Hamre offer an in-depth review of current standing on the influence of prenatal cannabinoid exposure on early development and beyond. The task is not trivial, as there is a clear difference of opinion between medical professionals and societal attitudes. The authors conclude that while there is evidence that prenatal cannabis exposure might alter developmental trajectories, the cause-effect relationships are not proven and, in most cases, require additional studies.

Strong focus in this Special Issue is maintained on vascular and cerebrovascular consequences of prenatal alcohol exposure. Momin et al. review vascular contributors to the neurobiological effects of prenatal alcohol exposure. This systematic review of the literature leads to a conclusion that although the brain has been traditionally considered as a main target of prenatal alcohol exposure, data from human samples and bench studies document that developing vasculature is equally sensitive to an early alcohol hit. Saha and Mayhan concur with this conclusion and expand upon it by presenting a review of mechanisms that underly cerebral vascular damage by prenatal alcohol exposure and which enable lifelong consequences of this adverse developmental event. Both reviews highlight the fact that our approach to treatment of adverse developmental outcomes should involve a multi-organ strategy.

Waite and Burd conclude the chapter of alcohol prenatal effects with a review of literature on common developmental trajectories and clinical identification of children with fetal alcohol spectrum disorders. They describe current challenges in the diagnosis of fetal alcohol spectrum disorders as opposed to prevalent behavioral disorders. Finally, Fleming et al (*in press*) offer an early, time-efficient screening tool which could assist in efforts to diagnose fetal alcohol spectrum disorders in large cohorts of children. Wide clinical utility of this newly offered tool remains to be documented.

Following current trends of increased opioid use, misuse, and opioid-related deaths, Madurai et al. describe alterations in the peripheral inflammatory and central immune landscapes following prenatal exposure of rats to methadone. The authors conclude that such alterations may underly long-term consequences of developmental brain injury including cognitive and attention deficits. However, identification of altered patterns may also serve as a biomarker and a therapeutic target. Translation of these findings from bench to bedside remains a goal for near future.

Author contributions

AB wrote a first draft. DA edited and added more detail. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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High-resolution imaging in studies of alcohol effect on prenatal development

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Fetal alcohol syndrome represents the leading known preventable cause of mental retardation. FAS is on the most severe side of fetal alcohol spectrum disorders that stem from the deleterious effects of prenatal alcohol exposure. Affecting as many as 1 to 5 out of 100 children, FASD most often results in brain abnormalities that extend to structure, function, and cerebral hemodynamics. The present review provides an analysis of high-resolution imaging techniques that are used in animals and human subjects to characterize PAE-driven changes in the developing brain. Variants of magnetic resonance imaging such as magnetic resonance microscopy, magnetic resonance spectroscopy, diffusion tensor imaging, along with positron emission tomography, single-photon emission computed tomography, and photoacoustic imaging, are modalities that are used to study the influence of PAE on brain structure and function. This review briefly describes the aforementioned imaging modalities, the main findings that were obtained using each modality, and touches upon the advantages/disadvantages of each imaging approach.

KEYWORDS

blood alcohol concentration, brain imaging, brain metabolism, maternal drinking, fetal development, alcohol *in utero*

Introduction

Globally, alcohol (ethanol) is the most widely used psychotropic drug (1). Depending on gender and different countries, the drinking levels of alcohol can be considered light, moderate, heavy, or binge drinking. Moderate drinking involves one drink for women and two drinks for men in a day (2). Binge drinking can be typically classified as 4 or more

Abbreviations: ARBD, alcohol-related birth defects; ARND, alcohol-related neurodevelopmental disorders; BAC, blood alcohol concentration; DTI, diffusion tensor Imaging; FAS, fetal alcohol syndrome; FASD, fetal alcohol spectrum disorders; fMRI, functional magnetic resonance imaging; fPAT, functional photoacoustic tomography; GD, gestational day; MRI, magnetic resonance imaging; MRM, magnetic resonance microscopy; MRS, magnetic resonance spectroscopy; PAE, prenatal alcohol exposure; PAT, photoacoustic tomography; PET, positron emission tomography; pFAS, partial fetal alcohol syndrome; sPAT, structural photoacoustic tomography; SPECT, single-photon emission computed tomography.

drinks for women or 5 or more drinks for men consumed within a couple of hours of each other (3) leading to a blood alcohol concentration (BAC) level of 0.08 g/dL and higher (4). Moreover, heavy drinking can be reported as 8 or more drinks for women per week and 15 or more drinks for men per week (5).

According to the World Health Organization (WHO) Global Status Report on Alcohol and Health, in 2018, it was estimated that the total consumption of pure alcohol was 6.4 L per individual 15 years or older worldwide (6). In the United States, an estimated 38.5 million adults indulge in binge drinking per month, among which adults aged 18–34 years hold the highest prevalence (26%) (7). Excessive alcohol intake can lead to a plethora of detrimental effects targeting multiple organs such as the brain, liver, pancreas, and heart (8–12). Moreover, it increases the chance of developing various pathological conditions that include chronic diseases, cancers, and mental disorders (13–17). In particular, women of reproductive age are reported to be frequent users of alcohol (18, 19). Even more alarming, estimated global alcohol consumption rate during pregnancy is ~9.8% (20). The WHO European Region points at an average of 25.2% alcohol consumption rate during pregnancy. This statistic involves countries like Russia, United Kingdom, Denmark, Belarus, Ireland, Italy, France, and Finland (20). Whereas the WHO Eastern Mediterranean region (Oman, United Arab Emirates, Saudi Arabia, Qatar, Kuwait) reports the lowest average alcohol use at 0.2% among pregnant women (20). While socio-demographic (e.g., age, ethnicity, education level, reporting conditions, religious affiliation) and socio-economic (e.g., employment, nutritional diet, and prenatal care) factors play an essential role in the variability of alcohol consumption estimates (21–24), alcohol use among pregnant women does not decline. Between 2018 and 2020, the prevalence of alcohol consumption among pregnant women in the United States increased to 13.5%, and 5.2% were involved in binge drinking (25). Considering the deleterious effect of alcohol on health, alcohol use during pregnancy does not only affect pregnant women themselves but also their fetuses.

Although many women tend to stop or reduce drinking levels of alcohol once diagnosed with pregnancy, a high rate of unplanned pregnancies (45%) (26) may cause prenatal alcohol exposure (PAE) unknowingly during the first trimester. Collectively with the reported statistics on alcohol consumption prevalence during pregnancy, it can be inferred that a significant number of fetuses are exposed to the toxic effects of alcohol with or without the knowledge of women that they are pregnant. Such astonishing statistics inevitably leads to a plethora of health complications associated with PAE. In this review, we will briefly describe health concerns arising from alcohol exposure *in utero*, obstacles in their therapeutic treatment, and challenges faced by contemporary drug discovery efforts. We will then highlight the need for high-resolution imaging tools that would aid in the research process for assessment of pathophysiology and identification of promising drug targets for successful treatment of consequences arising from PAE. Finally, we will describe current advancements made in the field of high-resolution imaging that can be used as stepping stones for visualization of alcohol-related damage in small laboratory animals. We will conclude with the prospects of using high-resolution imaging at the cross-over of physics and biology for successful diagnostics and treatment of PAE-related health disorders.

Health consequences of PAE

Alcohol is capable of easily and rapidly passing from the mother's bloodstream *via* the placenta into the developing fetal circulation where it penetrates through blood-brain barrier, and targets multiple critical fetal organs (4, 27, 28). Alcohol can directly target several mechanisms at different stages of gestation and enable the teratogenic effects (29). These effects include disruption of neuronal cell survival, proliferation, and growth pathways leading to apoptosis (30) in the early gestation period, neonatal microglial abnormalities causing neuroinflammation (31), interference with the cortical vascular network development (32), alteration of cardiac progenitor cells gene expression (33), and dysfunction of the hypothalamus-pituitary-adrenal axis (34). Maternal alcohol consumption can result, first of all, in apparent gestational complications such as spontaneous miscarriage (35), premature delivery (36), low birth weight (37), placental abruption (38), first or second trimester bleeding, intra-amniotic infection (39), and intrauterine growth restriction (40). Generally, higher BAC peaks of alcohol are associated with higher risks for adverse effects targeting physical, psychological, and behavioral development of the fetus (41, 42). Yet, based on a pregnancy cohort study from 8 metropolitan areas in the United States, it was found that every successive week of alcohol use led to an 8% increase in the risk of spontaneous abortion and did not correlate to the number of drinks consumed per week or to binge drinking (35). This underscores the significant fact that no known amount of alcohol is safe during pregnancy.

Fetal alcohol spectrum disorders (FASD) is the umbrella term that describes the detrimental effects of PAE and includes four distinct categories: fetal alcohol syndrome (FAS), partial fetal alcohol syndrome (pFAS), alcohol-related neurodevelopmental disorders (ARND) and alcohol-related birth defects (ARBD) (43). PAE causes lifelong consequences and allows FASD diagnosis mainly within four domains: the level of PAE, facial dysmorphology, growth deformities, and neurodevelopment retardation (44–48). However, not every neonate exposed to alcohol during gestation will develop FASD as it is estimated that only one in every 13 pregnant women exposed to alcohol would deliver a child with FASD (49). This could occur due to several factors such as the quantity,

frequency, and timing of alcohol exposure, maternal age, diet, genetic and epigenetic factors along with the influence of other substance abuse (50–52). Yet, FASD are highly preventable neurodevelopmental disabilities with an estimated global prevalence of 0.77% which would result in 630,000 children born annually with FASDs worldwide (49). Unfortunately, the mechanisms causing FASDs are poorly understood, and no known cure has been developed (53).

FAS is the most severe form of FASD including craniofacial dysmorphic features, prenatal and postnatal fetal growth restriction, neurodevelopmental abnormalities, and cognitive or behavioral impairment (54). The three fundamental facial features of FAS include short palpebral fissures, smooth philtrum, and thin vermilion border of the upper lip; the cranial features include smaller head circumference, structural brain anomalies, and abnormal neurophysiology and in some cases recurrent non-febrile seizures (55). Various studies have demonstrated that PAE decreases the bioavailability of glutamine and glutamine-related amino acids and hence hinders fetal development (56, 57). It is reported that 0.15% of live births result in FAS globally and this percentage rises in countries that are characterized by a higher consumption of alcohol during pregnancy (e.g., Belarus, Italy, Ireland, Croatia, and South Africa) (20). However, in the case of pFAS, only a few characteristic features of FAS are present such as facial dysmorphology, neurocognitive impairment, and either growth restriction or microcephaly (44).

ARND is the most prevalent yet difficult form of FASD to be diagnosed (58). ARND includes neurocognitive and behavioral impairments but lacks the presence of distinct FAS cranial and facial phenotypes, consequently remaining undiagnosed or misdiagnosed (44, 54, 58). PAE induces neurotoxic effects resulting in morphological or functional alterations of specific neuronal structures and brain circuits (59, 60). In an observational cohort study, it was found that moderate or binge drinking during pregnancy disrupts the cortical connectivity and impairs cognitive functions in children (61). Compelling evidence from various brain imaging and animal studies shows that PAE hampers cognitive function in various areas such as learning, memory, attention, speech development, vision, adaptive skills, and motor skills (62–67). Behavioral deficits observed include hyperactivity, impulsivity, poor social skills, aggressive behavior, and mood disorders (62, 67, 68). A dose-dependent prenatal alcohol exposure study done by Lees et al. (2020) found evidence of differences in cerebral and regional brain volume associated with psychological and behavioral problems among adolescents aged 9–10 years (69). Neuroimaging studies also show youths exposed to heavy maternal alcohol exposure with smaller cerebral surface area and irregular cortical thickness in comparison to unexposed youths (70–72). Attention deficit hyperactivity disorder has high comorbidity with FASD and has been found to have a 48% prevalence among children diagnosed with FASD (73). ARNDs are often missed due to features that can overlap with several different neurodevelopmental disorders or can often be credited to environmental or socioeconomic factors for behavioral deficits.

ARBD fall under the rarer spectrum of FASD which requires a history of PAE coupled with a major systemic malformation (44). This malformation includes cardiac (atrial septal defects, aberrant great vessels), auditory (neurosensory hearing loss), skeletal (radioulnar synostosis, vertebral segmentation defects, scoliosis), and ophthalmic (optic nerve hypoplasia, retinal vascular anomalies) or renal defect (horseshoe kidneys) (54). Among all the global congestive birth defects, it is estimated that 5% of the total cases are contributed by PAE (74–76). Indirect toxicity from alcohol metabolites (e.g., acetaldehyde) and impaired placental nutrition supply also lead to PAE-induced organ damage (77, 78). Congestive heart defects occur from acute, early alcohol exposure during the first gestation trimester in humans (54, 73). In an avian model study, the early co-administration of glutathione along with ethyl alcohol (ethanol) increased the percentage of embryos with normal hearts from 40% to 79% *via* inhibiting the action of PAE on reducing global DNA methylation (79). Studies have also shown PAE-induced alterations in neonatal lung development such as decreased lung mass and delayed lung maturation (80), inhibition of alveolarization and vascular development (81), and formation of hypoplastic lungs (82). There are experimental studies that show PAE deteriorates renal functions involving renal acidification, potassium excretion, and renal tubular cell use (83–85).

Despite the economic and public health burden, there are several obstacles to the diagnosis and treatment of health defects arising from PAE. Although early detection and intervention of PAE play an essential role in the prophylaxis of FASD, the lack of valid reliable methods for noting maternal alcohol exposure is an ongoing challenge. Although there have been several non-invasive methods such as passive surveillance systems, clinical studies, and meta-analyses, these observations largely depend on maternal self-report. Such self-reports can lack accurate assessment due to recall bias, societal stigma, and inconsistent screening. However, ethanol biomarkers can also be used as an early PAE detection tool. The direct metabolites of alcohol such as fatty acid ethyl esters (FAEE) in neonatal hair and meconium (86–88) and ethyl sulfate in maternal urine (86, 89) are present as distinct biological biomarkers. There are also several indirect metabolites of ethanol such as ethyl glucuronide in neonatal meconium or maternal hair (90–92) and phosphatidyl ethanol in maternal blood (93), although these indirect markers are less specific and indicative of alcohol exposure (86, 94). Still, no biomarker has been validated as a specific and sensitive diagnostic marker for PAE-induced toxic effects (86, 95). Clearly, there is an urgent need for bench studies that are aimed at better understanding of PAE pathophysiology and at finding markers and cures of deleterious consequences posed by PAE.

Laboratory animal models to study PAE

While studies in humans offer immediate translation into the wide-scale clinical practice, standardization of drinking patterns, doses, and timing within a large maternal population represent an impractical and ethically challenging task (96, 97). Human studies are also inconsistent due to variable factors like maternal age, diet, genetics, social status, and multi-substance use (51, 52). Animal models present an invaluable research tool to study the molecular mechanisms by which alcohol exposure hampers prenatal development. The use of various animal species such as non-human primates, pig, sheep, and rodents allow for manipulating the drinking pattern, dose, timing, and control for other confounding factors. However, each species has advantages and disadvantages for studies focusing on PAE. For example, non-human primates closely match the gestational period of humans in terms of neurodevelopment and allow fetal magnetic resonance imaging (MRI) to assess PAE effects (98). Nevertheless, non-human primates are expensive models that are scarcely available and involve longer gestation periods and singleton pregnancies. Ovine species are also used for preclinical studies of FASD due to equivalent fetal brain size and body weight to a human fetus and comparable gestational period (147 days) (99). However, ovine models are characterized by ruminal fermentation and differ from the human metabolic pattern following alcohol ingestion (1, 100). Large animals like pigs produce large litters, express voluntary alcohol consumption and similar rates of alcohol intoxication and excretion as humans (101). Yet they lack the advantage of introducing genetic manipulations which are widely available in small rodents. The latter are the most widely used versatile research models that allow invasive molecular mechanism studies of fetal alcohol exposure. Rats are commonly used for FASD studies and demonstrate the structural, developmental, and behavioral deficits as in humans (102–104). Rats are also preferred over mice for behavioral studies as they are calmer, more social, and easier to examine learning and executive function (105, 106). Mouse models are smaller in size, easier to maintain, have a shorter gestation period, and larger offspring production. With the use of modern technology, mice offer genetic modeling and are available as transgenic, knock-in, and knock-out strains. Another advantage of mouse models is the development of similar dysmorphic features of FASD as observed in humans. Various studies show these observations including craniofacial dysmorphology (107), brain abnormalities (108), growth restriction (109), and cognitive deficits (110, 111). The disadvantage of using rodent models is the difference in gestation length where the third-trimester fetal development in humans is analogous to the early postnatal period of rodents (112). As a significant amount of brain development occurs postnatally among rodents (113), many studies administer ethanol to neonate pups, but the mechanisms of absorption, metabolism and excretion are significantly varied in prenatal and postnatal periods (114, 115). However, the major disadvantage of mouse model is the small fetal size that makes non-invasive imaging studies of brain development and its alterations by PAE barely feasible. Overcoming this limitation is paramount for further advancement of the field as current understanding of the neurobiology and pathophysiology of PAE and its teratogenic effects has been rooted in neuroimaging technologies, which have allowed researchers to study structural, metabolic, and physiological abnormalities resulting from PAE.

High-resolution imaging techniques: Principles and major findings relevant to the field of PAE

High resolution imaging technologies could broadly be classified into structural neuroimaging technologies which identify neuroanatomical changes associated with PAE; functional neuroimaging technologies, which measure various neurophysiological signal changes associated with functional activities within various organs; and metabolic imaging modalities which detect various neurochemical changes by measuring the concentration of neurometabolites such as choline-containing compounds - which are markers of cell membrane stability and myelination, N-acetyl-aspartate (NAA)- which are markers of neuronal/axonal density and viability, and creatine/phosphocreatine, a marker of metabolic activities (116, 117) (Table 1). PAE mostly impacts the brain due to alcohol-related neurobiological damage in early development (118, 119). Thus, the brain is the most widely studied organ for the effects of PAE.

For the purpose of this review, we conducted a search in Google Scholar, PubMed, ScienceDirect and Web of Science for relevant literature using a combination of the following words: “prenatal alcohol exposure,” “neuroimaging,” “fetal alcohol spectrum disorder,” “FASD,” “fetal alcohol syndrome,” “magnetic resonance imaging,” “MRI,” “magnetic resonance spectroscopy,” “MRS,” “magnetic resonance microscopy,” “MRM,” “animal models,” “diffusion tensor imaging,” “DTI,” “functional MRI,” “fMRI,” “positron emission tomography,” “PET,” “single photon computed emission tomography,” “SPECT,” “photoacoustic tomography,” “functional.” Apart from the language, which was restricted to “English,” there were no restrictions in the date or subject of the study, and we examined each abstract to determine relevance of the literature. We further identified other studies by referring to the references of the studies obtained from the various databases. We ended up with a total of 71 articles for this review. Below, we describe the various neuroimaging modalities, in terms of their principles and major findings relevant to the field of PAE. We

TABLE 1 Classification of high-resolution imaging modalities based on functionality. Structural neuroimaging modalities are used to study neuroanatomical changes associated with PAE. Functional imaging modalities are used to study neurophysiological changes, specifically hemodynamic changes associated with PAE, while metabolic imaging modalities detect various neurochemical changes associated with PAE by measuring the concentration of neurometabolites.

Structural neuroimaging technologies	Functional neuroimaging technologies	Metabolic imaging technologies
• Magnetic resonance microscopy (MRM)	• Functional magnetic resonance imaging (fMRI)	• Magnetic resonance spectroscopy
• Diffusion tensor imaging (DTI)	• Single-photon emission computed tomography (SPECT)	• Single photon emission computed tomography (SPECT)
• Structural magnetic resonance imaging	• Positron emission tomography (PET)	• Positron emission tomography (PET)
• Structural photoacoustic tomography (sPAT)	• Multispectral photoacoustic tomography (fPAT)	

divide the modalities into three groups based on their use in studies of structural, functional, or metabolic effects of PAE. Table 2 summarizes the main finding of the various high-resolution imaging modalities in humans and animals.

Structural neuroimaging technologies

Magnetic resonance imaging technologies (MRI)

MRI is a safe, non-invasive imaging modality capable of producing detailed three dimensional structural and functional information of tissues properties (120). MRI uses a strong magnet and radio frequency waves to measure tissue property-dependent signals from protons (water) within the living organisms. Tissue properties like density, local environment, blood oxygenation, water movement as well as relaxation properties (T1, T2) may influence the signal detectable by MRI in various ways. When irradiated with radiofrequency energy, protons within the tissue are forced to swing out of equilibrium with the MRI field becoming misaligned with it due to their spin. When the radiofrequency energy is turned off, the protons quickly realign with the field, releasing electromagnetic energy in the process. The electromagnetic energy (signal) detected and the time it takes the protons to realign with the magnetic field (T1, T2) are used to generate images of the tissue. Advancements in technology has led to the development of custom coils and more powerful magnets, capable of generating magnetic fields of up to 7.0 T and higher (107, 121). Various dyes and nanoparticles have also been developed for use in imaging contrast enhancement, resulting in high resolution MRI referred to as Magnetic Resonance Microscopy (MRM) (122–124). Unlike routine structural MRI, the resolution in MRM is in the micron scale, typically less than 100 microns. Modern systems now support about 21–43 microns isotropic resolution, with scanning time in the order of 30–120 min per specimen (107, 121). The diameter of the bore of the magnet is only about 5 cm, thus limiting the size of the imaged specimen. As a result, MRM is typically used in studies involving small animals like rodent models of PAE (107, 121, 125). It allows for imaging of embryos, as young as 10.5 days postfertilization (123), with the ability to view images in all planes simultaneously for morphological assessment.

Sulik et al. (107, 121, 126, 127) have characterized the developmental stage-dependent effects of PAE in mice using MRM-based analyses of fetal and postnatal mice. Timed C57B1/5J pregnant dams received a vehicle (control group) or two daily doses of intraperitoneal injection of 2.8–2.9 g/kg ethanol (ethanol group), administered at 4 h intervals on gestational days (GD) 7 and 8. Previous studies have shown that ethanol exposure on GD7 when early gastrulation occurs in mouse embryos, leads to a spectrum of craniofacial dysmorphology consistent with FAS (176, 177, 107). Similarly, GD8 lies within the early neurulation stage, and ethanol exposure at this stage has been shown to cause structural brain abnormalities (127). Control and ethanol-administered mice were stage-matched and on GD17, MRM was conducted on the fetal mice at either 7.0 T or 9.4 T. The resulting 29 μm isotropic resolution images were reconstructed and later processed using ITK-SNAP, a 3D segmentation/visualization software (128). Linear and volumetric morphological analyses was conducted with 3D reconstructions of selected brain, head/face and body regions obtained, and compared between the control and ethanol-administered groups. According to the results, acute ethanol exposure on GD7 results in a spectrum of facial and central nervous system defects, the most severe of which includes holoprosencephaly. As shown in Figure 1, the facial abnormalities may range from a slightly narrowed nose (a closely approximated nostril) and a slightly diminished central notch to an extremely narrowed snout and complete absence of a nostril. Furthermore, compared to the control, the lower jaw is deformed and appears short and narrow (126).

Compared to the control group, fetuses affected by ethanol in a mild fashion have brains looking fairly normal, but with smaller olfactory bulbs and a narrower space between cerebral hemispheres. As the severity of the teratogenic effect increases, olfactory bulbs may disappear completely and the hemispheres become indistinguishable across the midline (107, 121, 126, 127). Other GD7 ethanol exposure-induced abnormalities include cleft palate, pituitary dysgenesis, aglossia, aqueductal stenosis and eye abnormalities ranging from slight microphthalmia to bilateral anophthalmia (107,

TABLE 2 High-resolution neuroimaging technologies used in the study of prenatal alcohol exposure.

Imaging modality (anatomical)	References/ subject	Subject (age)	Findings
MRM	(126)	Mouse	Linear and volumetric analysis of MRM images of GD7 showed craniofacial dysmorphology and brain abnormalities, the most severe being holoprosencephaly (HEP), volumetric reduction in telencephalic structures, increased lateral ventricular volume in HEP
	(107)	Mouse	GD8 exposure results in optic nerve coloboma, choanal atresia, narrowing of cerebral aqueduct and 3rd ventricle enlargement
	(127)	Mouse	GD8 results in disproportionate reduction in olfactory bulb, hippocampus, cerebellum, along with a disproportionate increase in the sepal region and pituitary glands
	(172)	Mouse	GD-9 ethanol exposed mice presented with increase septal region width and a decreased cerebellar volume, along with enlargement of all ventricles. Noticeable misshapen cerebral cortex, hippocampus, and right striatum
	(171)	Mouse	GD7-11 ethanol exposed mice presented with significant decrease in cerebellar volume, along with increase septal volume GD12-16 ethanol treatment resulted in reduced hippocampal volume, along with enlarged pituitaries, and high incidence of edema/fetal hydrops
	(175)	Rat	GD1-20 exposed rats presented with reduced brain and isocortical volumes as well as isocortical surface area and thickness
DTI	(143)	Humans (Adult males 18 and over)	Alterations in the corpus callosum, ranging from thinning, hypoplasia, and complete agenesis. Reduced FA and elevated MD
	(146, 147)	Humans (children) (8–18 years old, mean age 13)	Disproportionate reduction in volume of genu and splenium
	(145)	Humans (Children) (7–11 years old, mean 13.8)	Dislocation in posterior corpus callosum, correlated to the extent of facial dysmorphology
	(173, 174)	Humans Children, aged 9.7–13.7)	Decreased FA in posterior portion of inferior longitudinal fasciculus and in left middle cerebellar peduncles (White matter)
	(175)	Rat	High FA in cerebral cortex
sPAT	(150)	Mouse	Maternal ethanol consumption on GD-17 induces significant reduction in fetal brain vessel diameter (up to 31.25%) and vessel density (up to 25.1%)
		(Metabolic)	
MRS	(46, 48, 169, 170)	Monkey Rat	Reduction in levels of NAA/creatinine and NAA/Choline in multiple brain regions, notably parietal and frontal cortices, thalamus, cerebellar dentate nucleus, frontal white matter, and corpus callosum
		(Functional)	
fPAT	(150)	Mouse	Maternal ethanol consumption on GD-17results in up to 39.78% reduction in hemoglobin oxygen saturation in fetal blood vessels, indicative of significant ethanol induced hypoxia in fetal brain circulation
fMRI	(152)	Humans (14.5 years old) Go/No-Go tasks	Similar Go/no-go task performance between groups. PAEs showed greater BOLD response across in prefrontal and cortical regions, but less response in caudate nucleus activation

(Continued on following page)

TABLE 2 (*Continued*) High-resolution neuroimaging technologies used in the study of prenatal alcohol exposure.

Imaging modality (anatomical)	References/ subject	Subject (age)	Findings
	(151, 155, 156, 157)	Humans (Spatial working memory)	PAE children and adults showed overall less brain activity, but greater interior-middle frontal activity compared to controls during simpler activities
		Age matched Children (7–10) years old, Adults (18–33) years old	PAE showed greater BOLD response in frontal, insular, superior, middle, temporal, occipital, and subcortical regions
	(154)	Human adults (23.0 years old) (Arithmetic and number processing)	PAE exhibit lower accuracy but comparable reaction times, compared to controls
	(159)	Humans (10 years old) (Verbal working memory)	PAE showed increased activation in the left dorsal frontal, left interior parietal and bilateral posterior temporal regions
SPECT/PET	(164)	Human (20.6 vs. 22.8 years old) (Resting state)	Decreases in relative regional cerebral metabolic rates were found in 5 brain regions comprising thalamus and basal ganglia
	(161, 162)	Human (10.5 vs. 9.8-year old)	Significant brain volume reduction in PAEs
		Also (8.6 vs. 16 years old)	Reduced serotonin transporter binding in the medial frontal cortex and increased striatal dopamine transporter binding in PAEs
		(Resting state)	SPECT showed mild hypoperfusion of the left hemisphere (especially in parietooccipital and frontal regions) in PAEs
	(160)	Human (6–29 years old) and (29, 35 years old) (Resting state)	SPECT revealed at least 25% CBF reduction in the temporal region relative to the cerebellum

MRM, magnetic resonance microscopy; DTI, diffusion tensor imaging; MRS, magnetic resonance spectroscopy; sPAT, structural photoacoustic tomography; fPAT, functional photoacoustic tomography; fMRi, functional magnetic resonance imaging; FA, fractional anisotropy; MD, mean diffusivity.

121, 127). GD8 deformities noted in MRM scans include optic nerve coloboma, choanal atresia, narrowing of the cerebral aqueduct and third ventricle enlargement (107, 121, 126, 127).

Using MRM scans, regional brain segmentation and subsequent characterization of region-specific alterations and volumetric changes have equally been reported. Key amongst these findings in GD7 exposure models include volume reduction in telencephalic structures accompanied by increased lateral ventricular volume, mostly in fetuses with evident holoprosencephaly (126). GD8 exposure causes a disproportionate reduction in the volume of the olfactory bulb, hippocampus, as well as cerebellum, with a disproportional increase in the septal region and pituitary volumes (127).

Diffusion tensor imaging (DTI)

DTI is an emerging non-invasive MRI technology based on the measurement of the water molecule diffusions. The measured quantity is the diffusivity, a constant of proportionality that relates diffusive flux to concentration gradients (129). Due to the presence of numerous structures within tissue, the diffusion of water molecules is usually not isotropic. Thus, the measured diffusivity (diffusion tensor) is anisotropic, due to microscopic tissue heterogeneity (130). The diffusion tensor describes the diffusion of water molecules using a Gaussian model and results in a 3×3 symmetric positive-definite covariant matrix (131). The latter is capable of revealing the microstructural integrity of the white matter fiber tracts, enabling the quantification of subtle tissue changes affecting the integrity of the brain's neural networks and interregional information transfer (132). White matter integrity is essential for effective functioning of a host of complex cognitive processes such as normal executive functions, attention, and processing speed (133–135). DTI measures the overall direction of diffusion of water molecules along white matter fiber tracts to access the structure and organization of different brain areas (136, 137). Two key scalar metrics are typically obtained from DTI. Firstly, fractional anisotropy, a scalar value between 0 and 1 which quantifies the overall directionality of diffusion and variation in axonal integrity. Secondly mean diffusivity, which describes the rotationally invariant magnitude of the average diffusivity and may primarily reflect myelin breakdown, changes in cellular density and volume. High fractional anisotropy and low mean diffusivity values are associate with healthier white matter microstructure whereas low fractional anisotropy and high mean diffusivity values are indicative of pathological white matter (70, 138). In the absence of discernable facial dysmorphology, such as in mild cases of PAE, high resolution DTI has proven to be effective in detecting ethanol-induced abnormalities in the white matter fiber tracts and has been applied in humans and animal studies alike. Specialized data

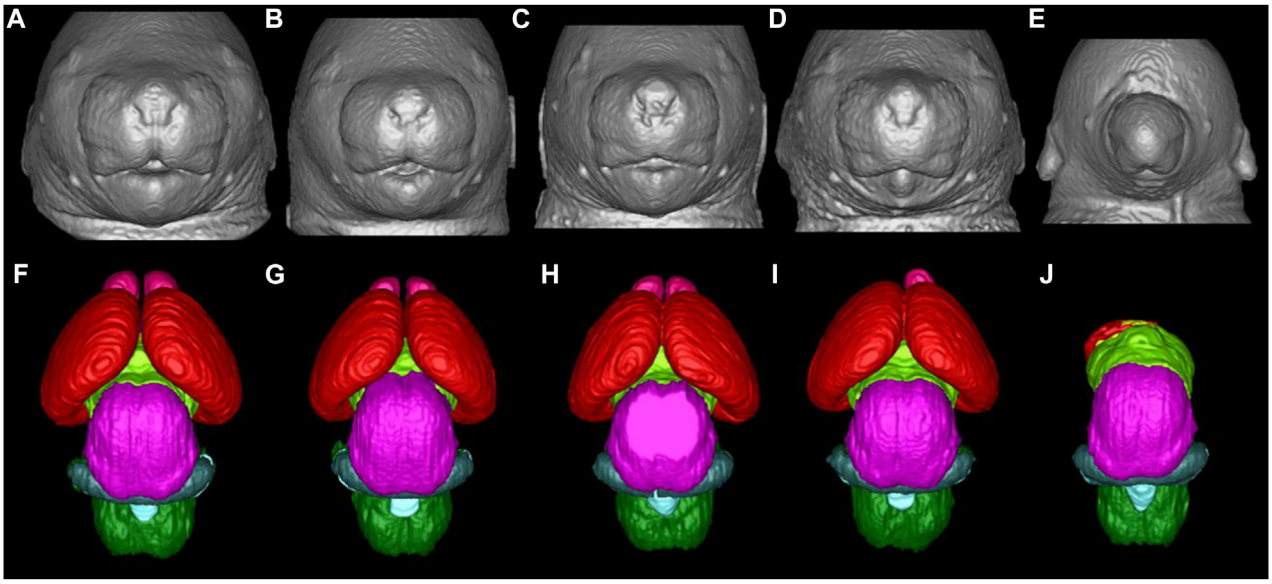

FIGURE 1
Facial and brain abnormalities following PAE on GD7 of the mouse. Compared to the control **(A)**, PAE-affected animals **(B-E)** show varying degrees of facial dysmorphology characterized by an elongated upper lip, a diminished philtra region, closely spaced nostrils, with small mandibles. The lower figures **(F-J)** show MRM-based 3D reconstructed brain anomalies from least to most severe. There is a correlation between facial dysmorphology and brain anomalies as animals with most subtle facial dysmorphology appear to have relatively normal brains. Animals with the more pronounced facial dysmorphology have a more severely affected brain, with the malformation corresponding to holoprosencephaly. **(E, J)**, is the most severe case with the brain completely missing most of its telencephalon with a severe facial phenotype, one nostril, with no lower jaw. Brain are color-coded as follows: olfactory bulbs (pink), cerebral cortex (red), diencephalon (lime green), midbrain (magenta), cerebellum (blue), mesencephalic/4th ventricle (teal), hindbrain (green). [Adapted from Ref. (11)].

analysis software such as DTI studio (139) and slicer3 (140) are used to create color-coded anisotropic maps from DTI data, to show the differing fiber orientation represented by the color-codes and the degree of diffusion anisotropy as represented by the signal intensity. DTI findings in human and animals (Figure 2) have revealed alterations in the corpus callosum, a structurally and functionally prominent brain commissural that actively connects the two cerebral hemispheres. These alterations (characterized by reduced fractional anisotropy and elevated mean diffusivity) range from complete agenesis of the corpus collosum to less severe alterations such as thinning and hypoplasia, with the thinning more localized in the posterior corpus collosum (141–145). Other quantitative studies have revealed disproportionate volume reduction in the genu and splenium of the corpus collosum of PAE subjects (146, 147). Sowell et al. (144, 145) identified dislocations in the posterior corpus collosum and correlated the degree of dislocation to the extent of facial dysmorphology.

Photoacoustic imaging for structural neuroimaging

In photoacoustic imaging, laser light is used to generate ultrasound waves from tissue, by irradiating the tissue with typically nanoseconds pulsed laser light (148). The most used wavelengths for tissue excitation are the visible and near intra-red region, typically in the range 532–1,100 nm, with the near infrared region from 600–900 nm offering penetration depths extending to several centimeters. Once the tissue is irradiated with sufficient light energy of the right wavelength to cause optical excitation, specific tissue chromospheres namely hemoglobin, lipids, water, melanin, etc., absorb the light energy, which is then rapidly converted to heat energy by vibrational and collisional relaxation, producing a small temperature rise within the surrounding tissues (148, 149). The rise in temperature produced by the energy deposition, typically less than 0.1 K induces a thermoelastic expansion, accompanied by an initial pressure rise, which launches a pressure wave within the surrounding tissue. The pressure waves propagate to the tissue surface where they are detected by an acoustic transducer as a sequence of time-resolved electrical photoacoustic signals called A-lines. Jiang and colleagues (150) used structural photoacoustic tomography (sPAT) to study the effects of maternal ethanol consumption on fetal brain blood vessel diameter and density in second-semester equivalent (GD17) pregnant CD-1 mice models of PAE. (Figure 3).

Jiang et al. (150) used structural photoacoustic tomography (sPAT) to study the effects of maternal ethanol consumption on fetal brain blood vessel diameter and density in second-semester equivalent (GD17) pregnant CD-1 mice models of PAE. PAT images were acquired for 40 min (at 5 min intervals) following

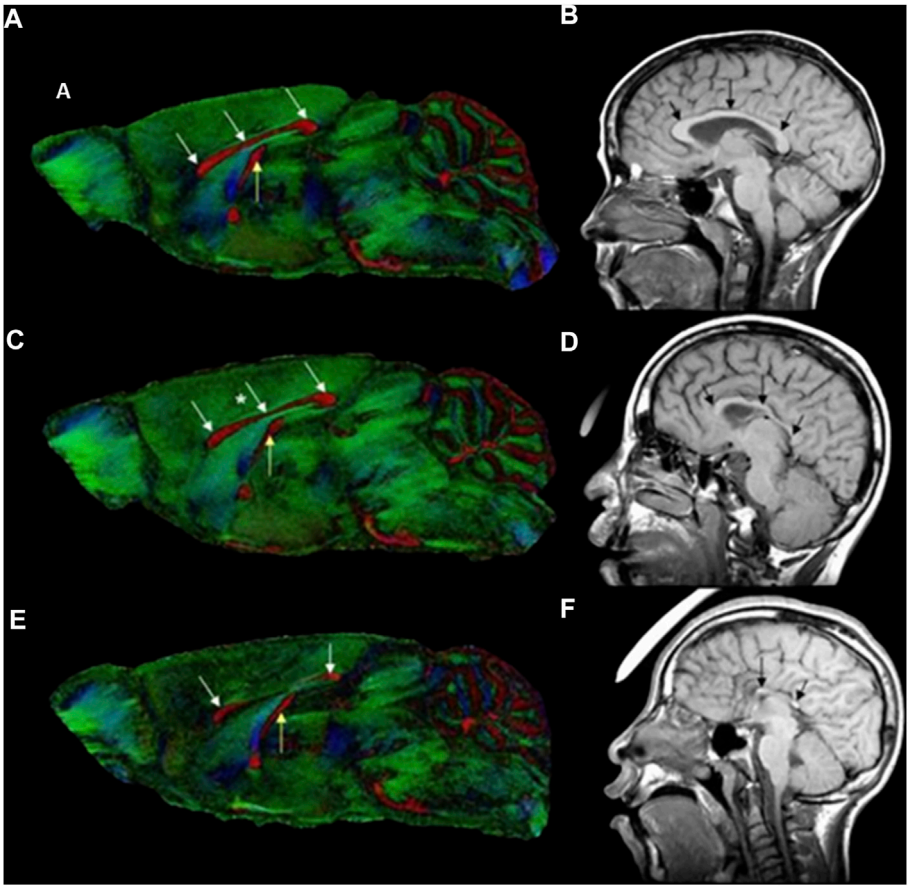

FIGURE 2
Color-coded fractional anisotropy maps from control mice **(A)** and GD7 ethanol exposed mice **(C, E)**, compared to a control individual **(B)** and FASD humans **(D, F)**. The ethanol exposed mice have varying degrees of brain dysmorphology compared to the control. The mouse in **(C)** has mild thinning of the corpus collosum in the middle section (*), while that in **(E)** has a reduced sized posterior and anterior corpus collosum with a completely absent middle part (see white arrows). The hippocampal commissure (yellow arrow) is also reduced in the more severely affected mice in **(E)**. The effect in mice is remarkably similar to that in humans with FASD. Compared to the control **(B)**, ethanol exposed humans **(D, F)** also have considerable dysmorphology of the corpus collosum (black arrows) [Adapted from (121)].

maternal intoxication of 20% ethanol at a volume of 3 g/kg *via* intraperitoneal injections. According to the results, maternal ethanol consumption on GD17 induces significant reduction in fetal brain vessel diameter (up to 31.25%) and vessel density (up to 25.1%)

Functional neuroimaging technologies

Functional neuroimaging techniques measure the neurophysiological signal changes in various brain regions that result from PAE. Signal changes of interest are typically collected from the subject when no specific task is occurring (such as during sleep—"Resting state"), when subjects perform a given task or when subjects switch between tasks. These results provide information about the neuronal mechanisms underlying brain functions associated with sensory and cognitive activities. Functional magnetic resonance imaging (fMRI), single-photon emission computed tomography (SPECT), and positron emission tomography (PET) are amongst the functional imaging modalities reportedly used to study the effects of PAE. Emerging technologies such as functional multispectral photoacoustic imaging have also been used in recent studies.

Magnet-based imaging modalities

Functional magnetic resonance imaging (fMRI) is a specialized form of MRI commonly used to study brain functions. Established in the early nineties, fMRI employs the difference in magnetic susceptibility between oxygenated and deoxygenated hemoglobin and the changes in concentration that results from local neural activation; to measure blood oxygen level dependent magnetic resonance signals. Local neural activation results in a corresponding localized increase consumption of energy, resulting in differential blood oxygen

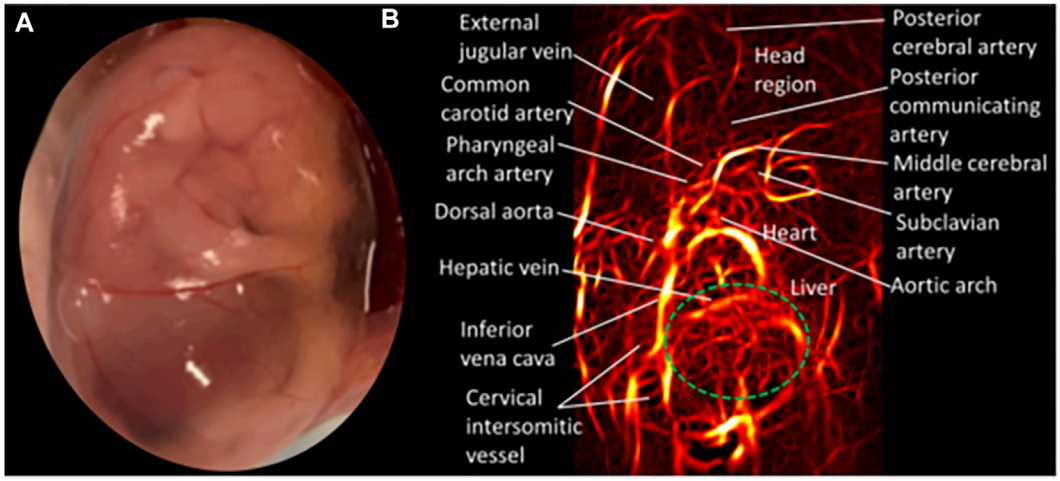

FIGURE 3
Structural photoacoustic tomography shows vascular tree of developing mouse embryoGD-17. **(A)** Photography of mouse embryo. **(B)** Photoacoustic image of fetal vasculature. Brain region within green oval shape [Adapted from (150)].

levels and thus a different blood oxygen level dependent signal for oxyhemoglobin and deoxyhemoglobin. fMRI is the most widely used functional imaging modality for studying the effects of prenatal ethanol exposure.

fMRI studies have examined functional changes in brain activity relating to specific cognitive tasks in subjects of PAE, compared to normal control subjects (Figure 4). Hemodynamic responses in subjects exposed to ethanol prenatally have been studied during various cognitive tasks including response inhibition (152, 153), mathematics and number processing (46, 154) working memory (151, 155–158) and verbal learning (159). Most of these studies report a difference in activation in the frontal regions between FASD subjects and controls. In go/no-go tasks, greater neural activation has been observed in several frontal and parietal regions during response inhibition in PAE subjects (152).

Radiation-based imaging modalities

Single photon computed emission tomography (SPECT) is a non-invasive functional imaging modality that uses gamma radiations to evaluate blood flow or concentration of various neurotransmitters. A radioisotope is injected into the organ of interest and a gamma camera is used to capture 2D projections of the organ with the distribution of radiotracers from different angles. A computer algorithm is then used to reconstruct the 2D projections into a 3D image of the organ of interest. SPECT is typically used to evaluate regional brain metabolic activities by coupling blood flow to regional brain metabolic activities. SPECT studies have identified differences in cerebral blood perfusion in the temporal (161), parieto-occipital, and prefrontal lobes (161) of prenatal ethanol exposed subjects, differences in medial-frontal serotonin transporter binding and increased striatal dopamine transporter binding in prenatal ethanol exposed subjects (162).

Positron emission tomography (PET) is a non-invasive functional imaging modality that uses radiopharmaceutical isotopes called radiotracers to visualize and measure physiological activities. Radiation emitted from radiopharmaceuticals injected intravenously into a subject is registered by external detectors positioned at different orientations. The radiopharmaceutical injected into the organ of interest breaks down and emits positrons, which interact with free electrons resulting in an annihilation reaction (163). The two photons (gamma rays) emitted from the annihilation reaction travel in opposite directions and arrive coincidentally at 180° to each other at the external detector. This signal is transferred to a computer for processing. PET is typically used to quantitatively evaluate glucose metabolism and blood flow associated with brain activity.

SPECT/PET studies are somewhat limited in their use to study the effects of prenatal ethanol exposure possibly because they focus on the "resting brain" and thus do no provide a direct insight into specific behavioral deficits. A PET study using PET/fMRI (see below) has identified differences in regional cerebral metabolic rates in the thalamus and basal ganglion between prenatal ethanol exposed subjects and normal subjects (164).

Photoacoustic imaging for functional neuroimaging

Multispectral photoacoustic imaging is a form of optical absorption spectroscopy (165) which attempts to identify the source of photoacoustic imaging contrast by exciting tissue at multiple wavelengths and identifying various contrast sources by means of their known optical absorption spectra. The selected wavelengths are such that the different absorber can be

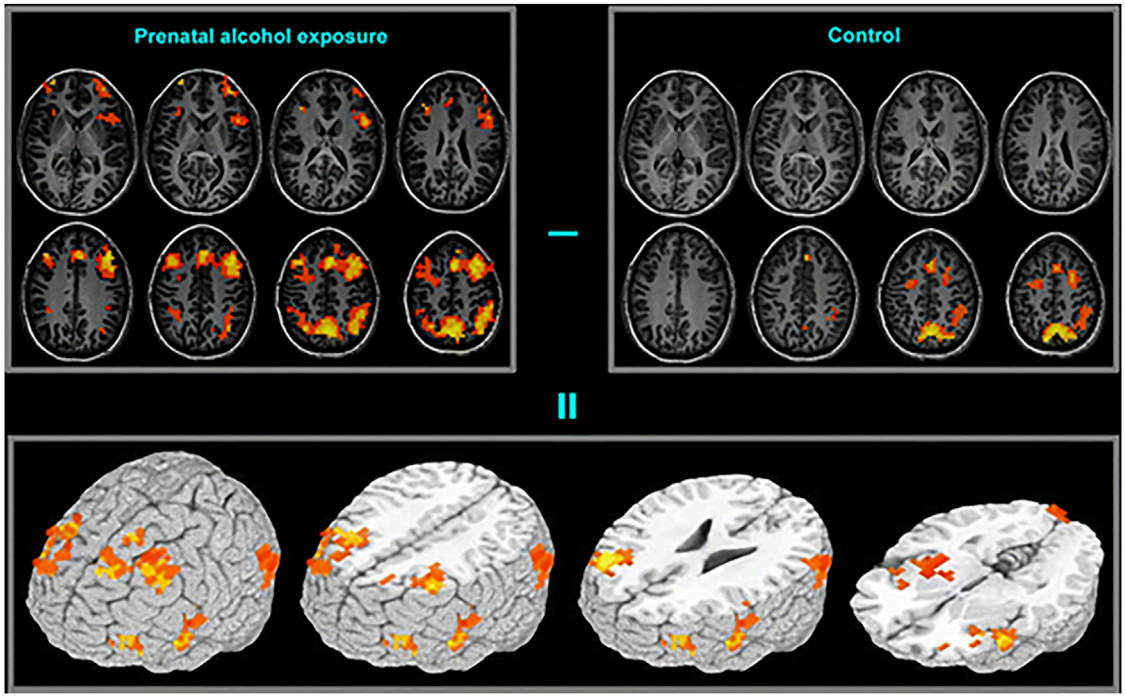

FIGURE 4
Functional brain activation differences (bottom frame) between the prenatal alcohol exposed (top-left frame) and control (top-right frame) subjects in a spatial working memory task. The exposed group exhibited greater activation in extended brain regions [Adapted from (151)].

distinguished from each other. After multi wavelength imaging, the resulting set of PAT images at each single wavelength are fed into a spectral unmixing algorithm, where they are converted to sets of images of specific absorbers.

Jiang and colleagues (150) extended their photoacoustic imaging study of the effects of maternal ethanol consumption on fetal brain blood vessel in second-semester equivalent (GD17) pregnant CD-1 mice models of PAE by using multispectral photoacoustic tomography (fPAT) to study ethanol induced oxygen saturation on fetal brain blood vessels (Figure 5). Multispectral PAT images were acquired for 45 min (at 5 min intervals) following maternal intoxication of 20% ethanol at a volume of 3 g/kg *via* intraperitoneal injections. The results show that, maternal ethanol consumption on GD17 induces up to a 39.78% reduction in hemoglobin oxygen saturation in fetal brain blood vessels, indicative of significant hypoxia in fetal brain circulation.

Modalities for imaging neurochemical (metabolic) effects of PAE

FASD studies in humans and animals typically use magnetic resonance spectroscopy (MRS) to study the metabolic effects of PAE. This involves studying changes in neurochemistry of various brain regions in PAE subjects and comparing the results to normal control subjects.

Magnetic resonance spectroscopy (MRS) is a non-invasive neuroimaging modality capable of providing biochemical information about specific brain regions (166, 167). When magnetic nuclei like ^{1}H, ^{31}P, ^{13}C or ^{19}F are placed in a magnetic field, they resonate at specific frequencies depending on the nuclei and the strength of the magnetic field. Thus, different radio frequency coils and hardware can be used to tune into these different frequencies to identify their origin. Due to the abundance in living tissue, and the strength of the magnetic resonant frequency, protons (^{1}H) are by far the most widely used nuclei for MRS. Protons, contained in various biochemical molecules in living tissue resonate at different frequencies depending on the electronegativity of the chemical bond they are involved in. Based on these frequency differences [typically measured in parts per million (ppm) due to the small size] in biochemical molecules (metabolites) can be distinguished (168). An MRS experiment typically involves exciting the nuclei in a specific volume of tissue with a radiofrequency pulse and receiving the resulting signal, in the form of a spectrum of signal intensity versus frequency, over a range of frequencies. These spectra can then be analyzed to identify the chemicals present in the volume as well as their relative concentrations if the peaks are suitably calibrated (168). Typical brain neurochemicals quantified by MRS include N-acetyl

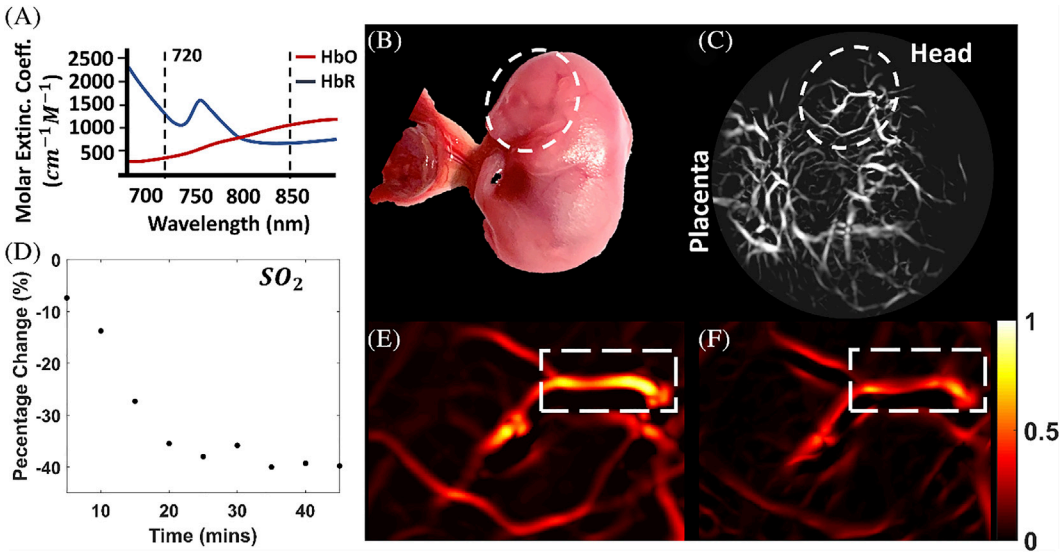

FIGURE 5
Multispectral photoacoustic imaging of oxygen saturation in fetal brain blood vessels following maternal ethanol intoxication on GD17. The multispectral data was acquired at the two wavelengths shown in **(A, B)**: Photograph of the mouse fetus. **(C)** Photoacoustic Images of the fetus. **(D)** Percentage change in oxygen saturation over time in selected blood vessels in **(E, F)**, oxygen saturation images [Adapted from (150)].

aspartate (a neuronal integrity biomarker), choline (an essential molecule for the synthesis of the neurotransmitter acetylcholine and cell membrane constituent phosphatidylcholine), and creatine (an essential component for maintaining energy-dependent systems in cells, gamma-aminobutyric acid, glutamate and myoinositol) (166).

O'Leary et al. (170) used an animal model of neonatal ethanol exposure to study regional brain neurochemistry in developing rats. They administered ethanol to offspring early during postnatal life to mimic third trimester ethanol exposure in the human and used a specialized MRS technique called high-resolution magic angle spinning to ascertain and quantify neurochemical data from intact brain biopsies. The results from spectral analysis showed that neonatal ethanol exposure results in region specific alteration in a number of neurochemicals including glutamate, N-acetyl-aspartate, gamma-aminobutyric acids etc., with the most pronounced alterations occurring in the cerebellum. The findings are consistent with earlier results by Green et al. (178), who observed reduced levels of N-acetyl-aspartate and taurine (an inhibitory neuromodulator) in the cerebellum of both male and female neonatal rats following binge ethanol exposure; with lower glutamate levels in females, compared to controls. Several other studies employing proton (^{1}H) MRS to study neurochemistry in PAE in humans and animals (46, 48, 168–170), have observed similar alterations in neurochemicals, with the most consistent results being a reduction in levels of neurochemicals like N-acetyl aspartate/creatine and N-acetyl aspartate/choline ratios in multiple brain regions, notably the parietal and frontal cortices, thalamus, and cerebellar dentate nucleus as well as the frontal white matter and corpus callosum (169).

Conclusion and prospects

While epidemiology data on prevalence of PAE and resulting brain-targeted effects of FASD are staggering, high-resolution visualization of morphological and functional parameters of the brain lags behind. Variants of MRI technologies including MRM, DTI, and MRS, as well as radiation-based PET and SPECT imaging, are amongst the modalities consistently used to study the effects of PAE in humans and animals. FASD studies in humans and animals using various structural neuroimaging modalities have revealed several distinct abnormalities in the developing fetus owing to PAE. While some imaging modalities are specific to animals, others could be used in both animals and human subjects. Development of easily accessible high-resolution imaging approaches, such as photoacoustic imaging, holds promise for early diagnosis and successful therapeutic interventions in the field of PAE.

Author contributions

Health risks of prenatal alcohol exposure (high prevalence, why do we have to study it?): ANB and ST. Laboratory models to study prenatal alcohol exposure: advantages and disadvantages of small rodents: ANB and ST. High-resolution imaging techniques: principles and major findings relevant to the field of prenatal alcohol exposure HJ and AMM. Concluding remarks and future of bioimaging in the field of prenatal alcohol exposure: ANB, HJ, and AMM.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Influence of prenatal cannabinoid exposure on early development and beyond

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Public perception surrounding whether cannabis use is harmful during pregnancy often diverges greatly from the recommendations of doctors and healthcare providers. In contrast to the medical guidance of abstinence before, during, and after pregnancy, many women of reproductive age believe cannabis use during pregnancy is associated with little potential harm. Legalization and social cues support public perceptions that cannabis use during pregnancy is safe. Moreover, pregnant women may consider cannabis to be a safe alternative for treating pregnancy related ailments, including morning sickness. Compounding the problem is a lack of medical and federal guidance on safe, low, or high-risk levels of cannabis use. These issues mirror the continuing debate surrounding alcohol use and health, in particular, whether there are safe or lower risk levels of alcohol consumption during pregnancy. Clinical studies to date suffer from several limitations. First, most human studies are correlative in nature, meaning that causal associations cannot be made between *in utero* cannabis exposure and health and behavioral outcomes later in life. Due to obvious ethical constraints, it is not possible to randomly assign pregnant mothers to cannabis or other drug exposure conditions—a requirement needed to establish causality. In addition, clinical studies often lack quantitative information on maternal exposure (i.e., dose, frequency, and duration), include a small number of individuals, lack replication of outcome measures across cohorts, rely on self-report to establish maternal drug use, and suffer from unmeasured or residual confounding factors. Causal associations between maternal cannabis exposure and offspring outcomes are possible in preclinical cohorts but there is a large amount of heterogeneity across study designs and developmental differences between rodents and humans may limit translatability. In this review, we summarize research from human and preclinical models to provide insight into potential risks associated with prenatal cannabinoid exposure (PCE). Finally, we highlight gaps in knowledge likely to contribute to the growing divide between medical guidance and public attitudes regarding cannabis use during pregnancy.

KEYWORDS

development, cannabinoids, prenatal, cannabis, THC

Introduction

Cannabis is the most frequently used illicit drug during pregnancy. Use by pregnant women has been increasing in parallel with legalization of cannabis for medicinal and recreational purposes and public perceptions that use of cannabis products is not harmful. Two large studies of self-reported cannabis use in pregnant women in the US from 2002 to 2017 [467,100 women; National Survey on Drug Use and Health, NSDUH (1)], and in Northern California from 2006 to 2016 [279,457 women; Kaiser Permanente Northern California (2)], reported an increase in cannabis use over time. Past-month use reported by pregnant women in the NSDUH study was 7% in 2016–2017, an increase of 3.6%, while past-month daily/near daily use by pregnant women was 3.4%, an increase of 2.5% (1). Over the course of the Kaiser Permanente study the prevalence of cannabis use by pregnant women increased by 2.9% (3). Notably, cannabis use by pregnant women was highest during the first trimester relative to other trimesters. In the NSDUH study, 11% and 5.3% of pregnant women in 2016–2017 self-reported first trimester past-month use and daily/near daily use, respectively, and lower cannabis use during later trimesters (4).

It is possible that legalization of cannabis for recreational purposes has contributed to increased cannabis use in both pregnant and non-pregnant women. This relationship has not been rigorously evaluated but numerous studies have found an inverse relationship between cannabis use and perceived risk [for review see (5)]. One recent study found evidence in support of an additive interaction between cannabis use and perceptions of cannabis as being low-risk and available (5). Pregnant woman also reported cannabis use to manage or relieve stress, depression, or nausea (6–8). Perceptions of cannabis as safe and having medicinal properties are easy to reinforce through social networks, media, and/or commercial messaging. In one study, researchers contacted dispensaries in the guise of pregnant women suffering from vomiting and nausea, a common first trimester ailment. Out of the 400 Colorado cannabis dispensaries contacted, 69% recommended the use of cannabis products to manage symptoms of morning sickness (9). In another study, over 30% of online media included the use of cannabis to manage nausea and vomiting (10).

In stark contrast to public perception, cannabis use during pregnancy and lactation is strongly discouraged by the American College of Obstetricians and Gynecologists (11), the American Academy of Pediatrics (12), and the Society of Obstetricians and Gynaecologists of Canada's (SOGC) policy (13). Another growing concern among the medical and research community is that the potency (THC level) of cannabis and derived products has been steadily increasing since the 1990s (14). The impact of maternal consumption of high potency cannabis on fetal development is unknown, but exposure to higher levels of THC may have adverse effects on development. The most common route of cannabis administration reported by pregnant woman is smoking blunts or joints (6). Relative to oral administration, inhalation is associated with faster adsorption and higher THC bioavailability, however, oral administration may result in more prolonged exposure to certain classes of THC metabolites [see (15) for review]. The precise impact of potency and patterns of maternal cannabis use on the developing fetus remains unclear. Regardless of the method of consumption, it is clear from human and preclinical studies that the major psychoactive component of cannabis, THC, crosses the placenta into fetal tissues where it has the potential to interfere with development [for review see (16) and (17–25)].

In this review we provide a brief overview of the endogenous cannabinoid system, its role in development, and possible disruption due to cannabis use. We also summarize research from human and preclinical models to provide insight into potential risks associated with prenatal cannabinoid exposure (PCE). Finally, we highlight limitations of the current research and areas where further research is needed.

Role of endogenous cannabinoid system in development

Exposure to cannabis during critical periods of development can interfere with homeostatic endocannabinoid system (ECS) function. The ECS plays a critical role in all stages of development, from fertilization, through adolescence, and beyond. Thus, exposure to cannabis has the potential to disrupt ECS function at nearly all stages of life. Below we provide a brief overview of the main components of the ECS and the potential impact of developmental cannabis exposure with a focus on brain development.

The main endogenous ligands of the ECS are the lipids N-arachidonylethanolamide (AEA or anandamide) and 2-arachidonoylglycerol (2-AG). AEA is synthesized from membrane precursors by N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and 2-AG is synthesized by 1,2-diacylglycerol (DAG) lipases DAGLα and DAGLβ. AEA is catabolized by fatty acid amide hydrolase (FAAH) whereas 2-AG is catabolized by monoacylglycerol lipase (MGLL). The primary effectors of the ECS are the G-protein coupled receptors (GPCRs) cannabinoid receptor 1 (CB1) and 2 (CB2). AEA has partial agonist activity at the CB1 and less so at the CB2. 2-AG acts as a full agonist at both cannabinoid receptors. AEA and 2-AG can activate other receptors, including GPCRs 55 and 119, and peroxisome proliferator-activated receptor (PPARs). AEA can also act as an agonist at transient receptor potential (TRP) channels (e.g., TRPV2, TRPV3, TRPV4, TRPA1, TRPM8). For a more detailed review see (26).

Direct disruption of the ECS by developmental cannabis exposure is thought to occur primarily through the binding of

THC to CB1 and CB2. TRP channels and several orphan G-protein coupled receptors (e.g., GPR55, GPR18) have also been shown to respond to THC and other cannabinoids found in cannabis (e.g., cannabidiol or CBD). However, their role in disruption of the ECS and developmental processes is less well understood. Indirect disruption of the ECS can also occur by altering the levels of endogenous cannabinoids. Very early in development, the endogenous endocannabinoids 2-AG and AEA and their receptors, CB1 and CB2 are alternatively expressed in a delicate spatial and temporal balance in reproductive tissue, uterus, placenta, and in the developing embryo and fetus where they play a collective role in fertilization, implantation, decidualization, and placentation [reviewed in (27)]. Moreover, use of cannabis prior to pregnancy and early in pregnancy (i.e., first trimester) could interfere with ECS homeostasis leading to infertility and adverse outcomes during pregnancy including inhibition of embryonic growth and miscarriage.

The ECS also plays a critical role in fetal brain development later in pregnancy (i.e., second and third trimesters). The binding of THC to CB1 and CB2 is known to disrupt neuronal development and connectivity (28–31). CB1 expression is evident in the developing human brain by 14 weeks and adult brain levels are reached by the end of the second trimester (24 weeks), albeit with regional expression differences apparent between fetal and adult brain (32). It is reasonable to assume that exogenous cannabinoid exposure, especially THC, during this time could interfere with cannabinoid receptor signaling and ECS function. Indeed, modulation of CB1 function during development in preclinical models results in disruptions in axonal pathfinding, progenitor cell expansion and neurogenesis, and specification of neuronal and glial cell lineages (33–35). Moreover, genetic deletion of CB1 in preclinical models is associated with altered morphology or function in numerous brain structures. These include cerebellum (36), cortex (37–39), striatum (40), bed nucleus of the stria terminalis (41), and other mesocorticolimbic areas (42).

Adolescence (12–18 years-of-age in humans and postnatal days 25 through 58 in rodents) is yet another critical period in brain development where the ECS plays a major role in the maturation and plasticity of corticolimbic brain regions [reviewed in (43)]. Adolescence is marked by neuronal circuitry maturation, synaptic remodeling and an overall reduction in synapse numbers, increasing white matter volume, and increasing cognitive capability (44). Prenatal exposure to THC may sensitize or subtly alter neuronal circuits leading to enhanced vulnerability and impairments that appear later in adolescence (45–47). For example, alterations in dopamine D2 (48) and μ opioid (49) receptors have been observed in human fetuses following prenatal cannabis exposure, although the duration of these alterations is unknown. In rodents, long lasting changes in dopamine (48, 50, 51) and opioid brain circuitry (specifically μ opioid receptor levels) have been observed along with increased seeking of heroin in adulthood (52, 53). Changes in adolescent behavior following prenatal THC exposure, including altered activity (54, 55), impaired memory (51, 56, 57), and inhibited social interactions and emotional reactivity (58, 59) have also been reported and will be reviewed in detail in later sections. Taken together, exposure to cannabis during pregnancy has the potential to disrupt the delicate balance of the ECS function and interfere with development. In the following sections we review birth and longer-lasting outcomes associated with PCE based on human and rodent research.

Clinical birth outcomes associated with PCE

Numerous studies (Table 1) in human cohorts have examined the potential relationship between PCE and birth or perinatal outcomes (i.e., low birthweight, stillbirth, preterm birth, neonatal distress, morphological defects, etc.). As mentioned previously, the ECS is important for implantation, placental development, and maintenance of the pregnancy [reviewed in (60)] and disruption of the ECS through exogenous cannabinoid exposure could exert direct effects on fetal development or indirect effects on intrauterine growth and survival.

The most frequently reported adverse birth outcome following PCE in clinical studies (Table 1) is low birth weight (62–66, 70–73, 76). Premature delivery (61, 63, 64, 71, 76) and admission to neonatal intensive care (64, 65, 71, 76) are also frequently associated with PCE. The studies reported in Table 1 often measure multiple birth outcomes, many of which are not significantly associated with PCE. In addition, several studies found no association between PCE and birth outcomes after correcting for potential confounding factors (67–69, 75). Most studies in Table 1 included a modest number (<1,000) of women with exposure to cannabis during pregnancy. A few studies increased this number several fold by leveraging information available through electronic medical health records (71, 76). Digital health information is a useful resource to evaluate the potential impact of PCE in larger populations but generally precludes analysis of cannabis exposure (i.e., frequency and use patterns) during pregnancy and may even select for individuals with heavy cannabis use.

Low birth weight, premature delivery, and admission to neonatal intensive care are the most frequently reported adverse birth outcomes significantly associated with PCE. Stillbirth and gross morphological defects were rarely associated with PCE, which suggests that the range of possible adverse outcomes following cannabis exposure may be relatively narrow. One important consideration affecting most of the studies in Table 1 is the inability to quantify dose, duration, frequency, and amount of exposure. In some studies, maternal exposure is classified by patterns of use (see Table 1), however the

TABLE 1 Birth outcomes.

Study	Region	Cohort	Exposed	Years	Cannabis use	Maternal use classification	Outcomes
(61)	South Australia	7,301 births	394	1975 to 1981	Self-Report	Nonuser Up to once per week (356) > once per week (36)	*Premature birth, fetal growth restriction*
(62)	Boston, United States	1,690 mother/child pairs	~230	1977 through 1979	Self-Report	Never Once per month (51) Once per month but < once per week (51) 1-2 times per week (101) 3+ timer per week (34)	*Low birth weight*
(63)	Connecticut, United States	3,857 mothers	367	1980 through 1982	Self-Report	Nonuser Occasional user (once per month or less; 158) Regular User (2-3 times per month or more; 209)	*Low birth weight, small for gestational age, premature birth (white women only)*
(64)	Ontario, Canada	98,512 women	5,639	2012 to 2017	Self-Report	No Yes	*Preterm birth, low birth weight, placental abruption, transfer to neonatal intensive care, and low Apgar scores*
(65)	8 countries	Meta-analysis of 24 studies	Variable	1982 and 2014	Self-Report	NA	*Low birth weight and transfer to neonatal intensive care*
(66)	Boston, United States	1,226 mothers and children	331	1984 through 1987	Self-Report and Toxicology	Negative Positive (For both self report and urinalysis)	*Low birth weight, decrease in body length*
(67)	7 University prenatal clinics across the United States	7,470 women	822	1984 to 1989	Self-Report and Toxicology	Negative Positive (For both self report and serum analysis)	Cannabis use during pregnancy not related to preterm birth or low birth weight
(68)	Boston, United States	12,424 women	1,246	August 1977 and March 1980	Self-Report	None Occasionally (880) Weekly (229) Daily (137)	Low birth weight, premature birth and major malformations more frequent for cannabis users but these were no longer significant after adjusting for confounding factors
(69)	Avon, United Kingdom	12,129 mothers	585 (use prior to pregnancy); 311 (use in 1st trimester), 250 (use mid pregnancy)	1991 through 1992	Self-Report	Never 6 months before pregnancy: Daily (109), 2-4 times per week (149), Once per week (49), <Once per week (278) 1st Trimester: Daily (61), 2–4 times per week (84), Once per week (34), <Once per week (132) Mid-pregnancy: Daily (53), 2–4 times per week (59), Once per week (39), <Once per week (99)	After adjusting for confounding factors, no outcomes were significant but there was a trend for negative association between frequency and duration of cannabis use during pregnancy and birth weight

(Continued on following page)

TABLE 1 (*Continued*) Birth outcomes.

Study	Region	Cohort	Exposed	Years	Cannabis use	Maternal use classification	Outcomes
(70)	Norway	9,312 women (10,373 pregnancies)	272	1999 and 2008	Self-Report	None Previous use before pregnancy (10,101) Curtailed use during 1 period in pregnancy (209) Prolonged use during at least two periods of pregnancy (63)	*Low birth weight*
(71)	United States	12,578,557 women and births	66,925	1999 to 2013	Electronic Medical Health Records	No diagnosis Diagnosis of cannabis dependence or abuse	*Intrauterine growth restriction, premature birth, stillbirth*
(72)	New York, United States	139 aborted fetuses	44	2000 to 2002	Self-Report and Toxicology	Non-users Before pregnancy: Nonuse (8), Light use (17), Moderate use (4), Heavy use (15) During pregnancy: Nonuse (8[a]), Light use (13), Moderate use (7), Heavy use (10)	*Reduction in body weight and foot length*
(73)	Rotterdam, Netherlands	7,452 mothers	459	April 2002 to January 2006	Self-Report	Nonuse Continued cannabis use (41) Cannabis use in early pregnancy (173) Cannabis use before pregnancy (245)	*Growth restriction of the fetus in mid- and late-pregnancy and lower birth weight*
(74)	Ohio, United States	325 mothers	111	2010 to 2015	Self-Report and Toxicology	No exposure Prenatal cannabis exposure (37) Prenatal cannabis and tobacco exposure (74) Prenatal tobacco exposure (66)	*Increased risk of small for gestational age (less than 10th and 5th percentiles)*
(75)	Ohio, United States	363 women	119	2010 to 2015	Self-Report and Toxicology	Negative Any positive (based on questionnaire, obstetrical record, or urine toxicology)	No association with preterm birth in this at-risk cohort
(76)	California, United States	3,067,069	29,112	2011 through 2017	Electronic Medical Health Records	No cannabis-related diagnosis Any cannabis-related diagnosis	*Premature birth, small for gestational age, admission to neonatal intensive care unit, major structural malformations (brain and gastrointestinal)*

Italicized text indicates significant associations with PCE. Exposed = Number of individuals within each cohort with cannabis use based on self-report or other measures. Maternal use classification defined in each study with the estimated number of individuals based on available summary tables. Light use = 0 > average joints per day <0.4; moderate use = 0.4 average joints per day <0.89, heavy use = average joints per day ≥0.89.
[a]self-reported non-use but positive toxicology test.

numbers in each category are frequently small and classification relies on self-report. Moreover, the composition of cannabis has changed substantially over the duration of these studies. In the US, the amount of THC in cannabis (i.e., potency) has tripled from ~4% in 1995 to ~12% in 2014 while the CBD content has decreased from 0.28% in 2001 to less than 0.15% in 2014 (14). In

the Netherlands, potency is likely to be even higher with THC levels of 17.7% reported for Dutch cannabis products (77).

Differences in cannabis composition and potency complicate comparisons of outcomes between studies. It is unclear whether the steep increase in cannabis potency is associated with any increases in birth outcome severity (Table 1). This issue has not been extensively explored, partly due to the inability to accurately quantify fetal exposure over the duration of the pregnancy and it remains unclear how potency and level of maternal cannabis exposure contribute to the risk of adverse birth outcomes. It is also important to acknowledge that associations found between cannabis exposure in pregnancy and birth outcomes in Table 1 do not imply causality. Other limitations include a reliance on self-report to assess fetal exposure, different comparison groups and control of possible confounding variables among studies, generally small sample sizes and possible selection bias (i.e., study individuals are not representative of the general population), and potential confounding by unmeasured or residual variables (e.g., socioeconomic status, maternal and fetal genetic factors, maternal behavior and nutrition, polydrug exposure, etc.).

Long-lasting impact of PCE on behavior and human brain development and function

Given the ubiquitous role of the ECS in development, from fertilization through adolescence, a reasonable hypothesis is that prenatal exposure to cannabis will have a long-lasting impact on child development. Below we summarize the research addressing this assertion. Most of the research into the potential long-term behavioral consequences associated with maternal cannabis use is based on population-based longitudinal cohorts where healthy mothers are recruited from the population and both mothers and offspring followed up at regular intervals.

Human longitudinal cohorts

The Maternal Health Practices and Child Development Study (MHPCD) and Ottawa Prenatal Prospective Study (OPPS) are the most comprehensive longitudinal studies yet completed. The OPPS (78) cohort primarily consisted of low-risk, middle-class white Canadians and included a total of 49 offspring tracked annually between birth and the age of 6, followed by less frequent follow ups through 22 years-of-age. The high-risk MHPCD (79, 80) cohort consisted of 763 mother-infant pairs at inception and included high-school-educated mothers of lower socioeconomic status and mixed ethnicity (52% African Americans and 48% European Americans) with light-to-moderate use of cannabis, alcohol, and nicotine living in the Pittsburgh, PA area. Cannabis and other drug use was assessed by self-report before pregnancy, at each trimester, and during subsequent follow-up interviews at 8 and 18 months and 3, 6, 10, 14, 16, and 22 years postpartum.

The largest international longitudinal cohort, with ~100,000 pregnant women recruited between 1998 and 2008, is the Norwegian Mother and Child Cohort Study (MoBa). Biological samples and questionnaire data were collected starting at 17 weeks of gestation. This low-risk and healthy lifestyle cohort has resulted in ~270 published studies to date. However, as of 2022, only a single study on birth outcomes related to PCE (70) (Table 1) has been published. Additional longitudinal cohorts are in progress.

Other ongoing studies include the Adolescent Brain Cognitive Development (ABCD) study, the Generation R study (GenR), and the Lifestyle and Early Achievement in Families study (LEAF). The largest US cohort is the ABCD study, which consists of ~12,000 US children enrolled between the ages of 9 and 10 at 21 different sites. The ABCD participants will be tracked for 10 years. The GenR study (81) enrolled 9,778 pregnant women with delivery dates from April 2002 to January 2006. These women were of higher socio-economic status and primarily from Dutch, Surinamese, Turkish and Moroccan ethnic groups in Rotterdam in the Netherlands. Outcomes were measured in early pregnancy (73; Table 1) with some offspring assessed through young adulthood. Behavioral outcomes at birth through age 6 are currently available. The LEAF study population consists of a high-risk cohort of pregnant women (63% African American) enrolled into the Ohio Perinatal Research Repository and includes 362 offspring (116 with prenatal cannabis exposure) eligible for continued follow-up from 3.5 to 7 years beginning in September of 2016 and continuing through August of 2020 (82). Unlike the other cohorts, LEAF study participants provided clinical samples (blood and urine) at enrollment and at each trimester. Thus, cannabis exposure based on THC metabolites can be assessed prospectively for this cohort. Findings from these longitudinal studies are discussed below.

Cognitive deficits

Cognitive ability and executive function span many dimensions including intelligence, achievement, comprehension, memory, attention, and impulse control. Cognitive outcomes associated with PCE in the various longitudinal cohorts are summarized below and in Table 2.

OPPS

Major deficits in cognitive function are not consistently detected across developmental stages in the OPPS cohort. A significant association with PCE was not detected until age 4, at which point maternal cannabis use was associated with lower verbal and memory domains assessed as part of the McCarthy Scales of Childrens' Abilities (MSCA) (83). These associations

TABLE 2 Alterations in cognitive function related to PCE in human longitudinal cohorts.

Cohort	Infancy (0 to 12 months)	Early childhood (1 to 4 years)	Middle childhood (5 to 8 years)	Preadolescence (9 to 12 years)	Adolescence (13 to 17 years)	Young adult (18 to 22 years)
OPPS	(95, 96) *Poor habituation to visual stimuli, increased tremors and startles (BNAS)* at 4 days old (97). *Increased fine tremors, Moro reflex tremors, startles, heightened motor reflexes (PNE, 9 and 30 days old) and increased hand to mouth behavior* (9 days old)	(98) No adverse effect on mental, motor, visual, or language outcomes (ages 1 and 2, BSID and RDLS) (83). No difference in verbal, quantitative, general cognitive, memory, and motor ability or language comprehension (MSCA/RDLS (age3) (83). *Lower verbal ability and memory subscale associated with heavy prenatal use* (MSCA and PPVT-FL, age 4)	(88) No difference in global intelligence, or cognitive and language ability or memory (aged 5 and 6 years, MSCA and PPVT-FL) (93). No difference on impulse control, deficit in sustained attention (age 6) (93) *Higher parental ratings of impulsivity/ hyperactivity* (age 6)	(87) No difference in reading and language measures on comprehensive test battery including WISC-III and WRAT (aged 9–12) (86). No difference in global intelligence or verbal ability but *decreased visual analysis and hypothesis testing and decreased impulse control* (WISC-III, Category Test, GDT age 9–12) (90). No difference in visuoperceptual function (TVPS). *Decreased performance in visual problem solving associated with heavy prenatal use* (WISC-III, age 9–12)	(85) No difference in most tests related to general intelligence, achievement, memory, and executive function. *Deficit in visual memory, analysis, and integration tasks* (age 13 to 16, WRAT, PIAT, MN, AD, SMT, KCT, WCST, Stroop, WISC-III) (89). *Less consistency in reaction times and more omissions indicative of a deficit in attentional stability* (age 13 to 16, CPT, WCST, ST, WISC)	(94) *Higher impulsivity and increased activity in bilateral PFC and right PMC and attenuation of activity in left CB during response inhibition* (age 18 to 22, Go/NoGo, fMRI) (91). No differences in visuospatial working memory (V2B). *More activity in left inferior and middle frontal gyri, left parahippocampal gyrus, left middle occipital gyrus, and left cerebellum. Less activity in the right inferior and middle frontal gyri* (fMRI, age 18–22) (92). No difference in task performance (V2B, Go/ NoGo, L2B, CST) *but changes in blood flow during tasks were altered, specifically, increased activity in posterior brain regions* (fMRI, ages 18–22)
MHPCD		(99) *Lower SBIS composite score, lower verbal reasoning and short-term memory deficits* (age 3)	(79) *Lower SBIS composite score, lower quantitative and verbal reasoning and deficits in short-term memory* (age 6) (100). *Higher impulsivity on CPT* (age 6)	(101) *Poor performance WRAT-R reading and spelling scores and PIAT-R reading comprehension score. Higher rate of educational underachievement* (age 10) (102). *SNAP attention deficits, hyperactivity, impulsivity* (age 10) (110). *Deficits in design memory and screen score* (age 10, WRAML)	(103) *Decreased WIAT composite and reading scores* (age 14) (104). *BCT decreased performance on one measure of processing speed and two measures of interhemispheric coordination and better performance on one measure of visual-motor coordination* (age 16)	(105) *Indirect effect on adult memory (WMS-III) mediated through intelligence (age 6), memory (age 10), and early-onset cannabis use (age 22)*
GenR		(106) *Attention problems in females* (CBCL, age 18 months)	(107) *Thicker frontal cortex with no change in gray or white matter volumes* (MRI, ages 6–8)			
LEAF		(108) No difference in executive functioning (age 3.5)				
ABCD				(109) No cognitive deficits or changes in brain activity (fMRI) during tasks measuring response inhibition (SST), reward processing (MID), and working memory (EN-Back). *Higher attention problem score* (CBCL, ages 9–10)		

Italicized text indicates significant associations with PCE. PNE, Prechtl neurological examination; BNAS, Brazelton Neonatal Assessment Scale; BSID, Bayley Scales of Infant Development; RDLS, Reynell Developmental Language Scales; MSCA, McCarthy Scales of Childrens' Abilities; PPVT-FL, Peabody Picture Vocabulary Test-Form L; SBIS, Stanford-Binet Intelligence Scale; CPT, Continuous Performance Task; MRI, Magnetic Resonance Imaging; WISC-III, Wechsler Intelligence Scale for Children; WRAT, Wide Range Achievement Test-Revised; Category Test, test of abstraction or concept formation ability; GDT, Gordon Diagnostic Delay and Vigilance Task; TVPS, Test of Visual-Perceptual Skills; PIAT-R, Peabody Individual Achievement Test-Revised; SNAP, Swanson, Noland, and Pelham attention subscale; fMRI, functional MRI; SST, Stop Signal task; MID, Monetary Incentive Delay task; EN-Back, EN-Back task of working memory; CBCL, Child Behavioral Checklist; PIAT, Peabody Individual Achievement Test; MN, Missing Numbers test of auditory and visual memory; AD, Abstract Designs test of auditory and visual memory; SMT, Sentence Memory Test of auditory and visual memory; KCT, Knox Cube Test of auditory and visual memory; WCST, Wisconsin Card Sorting Test; Stroop, Stroop Color/Word Interference Test; WIAT, Weschler Individual Achievement Test; BCT, computerized Bimanual Coordination Test; PFC, Prefrontal Cortex; PMC, Premotor Cortex; CB, Cerebellum; V2B, Visuospatial 2-Back task; Go/NoGo, Go/NoGo task; L2B, Letter 2-Back task; WMS-III, Weschler Memory Scale-3rd Edition.

were no longer significant at later developmental periods following adjustment for potential covariates (84–88). During preadolescence (i.e., ages 9–12), some aspects of visual-motor integration, non-verbal concept formation, and visual problem solving were significantly associated with PCE (86, 89, 90). Some of these PCE associated deficits (e.g., alterations in visual memory, analysis, and integration) persisted through adolescence (85). However, in young adults (i.e., ages 18–22), PCE was no longer found to predict deficits in visuospatial working memory (e.g., Visuospatial 2-Back or V2B) (91). Long lasting alterations in brain function may still be associated with PCE in the absence of profound deficits in cognitive function. Young adults with PCE showed significant and differential activation of brain regions during task performance (e.g., V2B, Go/NoGo, Letter 2-Back or L2B, Counting Stroop Test or CST) as measured by functional magnetic imaging (fMRI) even though task performance was similar between individuals with and without PCE (91, 92).

In contrast, deficits in attention and impulsivity associated with PCE were identified at multiple developmental stages in the OPPS cohort. Sustained attention deficits were significantly associated with PCE at age 6 (93) as were higher parental ratings of impulsivity and hyperactivity (93). During preadolescence, PCE was associated with decreased impulse control (86). During adolescence (i.e., ages 13–17), mild attentional deficits were significantly associated with PCE (89). In young adults, higher levels of impulsivity and alterations in cortical and cerebellar brain activity during response inhibition were significantly associated with PCE (94).

MHPCD

Potential cognitive issues related to PCE exposure appeared earlier in development and were more consistently detected for the higher risk MHPCD cohort. At the 3-year follow up during early childhood, maternal cannabis use in the second trimester was significantly and negatively correlated with short term memory subscale scores on the Stanford-Binet Intelligence Scale (SBIS) (99). In African Americans, first trimester use predicted a lower score on the verbal reasoning subscale and second trimester use predicted a lower score on the short-term memory subscale. In European Americans only, preschool attendance counteracted the negative impact of PCE on short-term memory and verbal reasoning. Cognitive deficits persisted at the 6-year follow up during middle childhood and problems of attention and impulsivity emerged. At this age, lower intelligence test composite scores were associated with heavy use (i.e., maternal use of one or more marijuana cigarettes per day) (79). Heavy first trimester cannabis use predicted lower verbal reasoning scores while heavy second trimester use predicted lower composite, short-term memory, and quantitative reasoning scores. Heavy use in the third trimester also predicted lower quantitative reasoning scores. Second trimester cannabis use also predicted higher impulsivity scores (i.e., greater errors of commission on the CPT-3) and, counterintuitively, higher attention scores (i.e., fewer errors of omission on the CPT-3) (100).

However, at the 10-year follow up during preadolescence there appeared to be little impact of PCE on most neurocognitive domains tested [e.g., problem solving and abstract reasoning (computerized Wisconsin Card Sorting test or WCST); learning and memory (WRAML); attention, visuomotor tracking and problem solving (Trail Making, Parts A and B), mental flexibility (Stroop Color/Word Interference Test), psychomotor speed and eye-hand coordination (Grooved Pegboard), attention and mental efficiency (Continuous Performance Test), attention, impulsivity, information processing efficiency and motor control (The Pediatric Assessment of Cognitive Performance Test] (110). Notable exceptions included impulsivity, reading comprehension and educational achievement. Second trimester maternal cannabis use predicted higher impulsivity (albeit near the end of the CPT task in the 3rd trial) (110) and was also a significant predictor of reading comprehension scores, teacher evaluations, and under-achievement (a mismatch between ability and teacher-rated academic achievement) (101).

Access to individual longitudinal data across many behavioral outcomes in the MHPCD enabled advanced statistical approaches to evaluate causal interactions and potential mediation between PCE, study outcomes, and both measured and unmeasured variables. Structural equation modeling (SEM) found that first trimester PCE and school achievement at age 10 were both mediated by child psychological status, which was independent of PCE (101). The indirect impact of PCE on adult memory was also assessed by SEM at the 22-year follow up in young adulthood (105). Although the authors found no evidence of a direct effect of PCE on adult working memory, they reported an indirect effect on adult memory mediated through intelligence (assessed at age 6), adolescent memory (assessed at 10 years), and early-onset (initiation at < 15 years of age) cannabis use.

Other cohorts

The GenR, LEAF, and ABCD studies also reported attentional deficits associated with PCE, but few cognitive problems following PCE. In the GenR cohort, PCE was associated with attention problems in 18-month-old females as measured using the Child Behavior Checklist (CBCL) for toddlers (106). The LEAF study was specifically designed to test the influence of PCE on executive function (i.e., inhibitory control, attention, planning ability, cognitive flexibility, episodic memory, processing speed, working memory, visual-spatial ability, and emotional regulation) and aggressive behavior in early to middle childhood (82). However, at age 3.5 there was no difference in executive functioning between children with PCE and unexposed children (108). Likewise, during preadolescence (i.e., ages 9 and 10) in the ABCD cohort,

cognitive deficits and changes in brain activity (fMRI) during tasks measuring response inhibition (i.e., the Stop Signal Task or SST), reward processing (i.e., the Monetary Incentive Delay task or MID), and working memory (i.e., the En-Back test) were not associated with PCE (109). However, PCE was significantly associated with higher attention problems (CBCL, ages 9–10) (109).

Brain morphological changes

Imaging studies in these human longitudinal cohorts are beginning to address whether changes in brain function and morphology during development are associated with PCE. Global brain regional volume was assessed by magnetic resonance imaging scans (MRI) in a subset (i.e., 96 children with prenatal tobacco exposure, 54 children with PCE, and 113 unexposed children) of the GenR cohort during middle childhood (i.e., 6–8 years) (107). In this imaging study, PCE was significantly associated with thicker frontal cortices relative to unexposed controls, while changes in gray or white matter volume were not associated with PCE. These findings suggest that children with PCE may undergo delayed cortical maturation and cortical synaptic pruning, a tantalizing result given that cognitive deficits appeared more prominent at earlier developmental stages in several cohorts (e.g., OPPS, MHPCD). However, the association between PCE and cortical morphology is correlative in nature and needs to be examined more rigorously to establish causality, developmental timing, underlying mechanisms, relationship to behavioral outcomes, and reproducibility.

Psychopathology and externalizing/internalizing behavioral problems

Aggressive behavior, externalizing behavior, and increased psychopathology have been significantly associated with PCE in several cohorts and are summarized below. However, family environment or genetic factors also appear to contribute to these traits.

Externalizing problems

Aggressive behavior (as measured using the CBCL for toddlers) in 18-month-old females was significantly associated with PCE in the GenR cohort (106). Aggressive behavior was also significantly higher in 3.5-year-old children with PCE relative to non-exposed children in the LEAF cohort (108). Numerous behavioral problems (i.e., higher withdrawal symptoms, externalizing behavior problems, and oppositional defiant behaviors) reported by a mother or caregiver were also significantly associated with PCE at age 3.5 in the LEAF cohort (108). Later, in middle childhood (i.e., age 6), PCE was associated with a higher level of hair cortisol concentrations relative to non-exposed children, suggestive of alterations in child HPA-axis function following *in utero* cannabis exposure (111). In the ABCD study, PCE was significantly associated with higher externalizing problems and higher total problem scores (CBCL) (109) during middle childhood (i.e., ages 9 and 10). In the GenR cohort, during both middle childhood and preadolescence (i.e., ages 7 through 10), PCE was associated with offspring externalizing problems (112). However, maternal cannabis use prior to pregnancy and paternal cannabis use were also associated with child externalizing problems suggesting that these associations are not due solely to *in utero* cannabis exposure. Shared familial and/or genetic confounding factors or additional residual or unmeasured confounding factors may have contributed to the observed associations in the GenR cohort (112).

Internalizing problems

For the MHPCD cohort, at the 10-year follow up, first and second trimester cannabis exposure was significantly associated with higher levels of depressive symptoms based on child self-report (113). However, a subsequent analysis of combined depression and anxiety symptoms at the 10-year follow up found only a marginal association between PCE and levels of self-reported depression/anxiety symptoms (114).

Psychopathology

At the 10-year follow up in the GenR cohort, increased psychotic-like experiences were significantly associated with PCE, maternal cannabis use prior to pregnancy, and paternal cannabis use (115). These associations are highly suggestive of multiple shared etiologies for psychopathology. Psychosis proneness (total score on Prodromol Questionaire-Brief Child Version) was also measured in preadolescents (i.e., age 8.9 through 11) in the ABCD study. Continued use of cannabis after knowledge of pregnancy was significantly associated with increased child psychosis proneness (116) and increased psychotic-like experiences (117) and greater psychopathology (i.e., higher CBCL scores for psychotic-like experiences and internalizing, externalizing, attention, thought, and social problems) (117). In the ABCD cohort, longitudinal analysis of children aged 8.9–13.8 years found that maternal use of cannabis after knowledge of pregnancy was associated with persistent vulnerability to psychopathology during the period of preadolescence (118). PCE was also associated with an increased frequency of psychotic symptoms in young adults from the MHCP cohort (119).

Sleep alterations

Alterations in sleep following PCE have been reported for different ages in several cohorts. A small sleep pattern sub-study

(20 controls and 18 cannabis exposed children) of 3-year olds from the MHPCD cohort found that PCE was associated with frequent nocturnal arousals after sleep onset, more awake time following onset of sleep, and lower sleep efficiency without any change in overall sleep duration or time spent in each sleep stage (120). Analysis of sleep patterns at 3.5 years of age in the LEAF cohort found that PCE was significantly associated with more sleep-related problems based on maternal or caregiver reports (108). A recent analysis of over 11,000 children aged 9–10 years enrolled in the ABCD study (242 with likely continuous cannabis exposure during pregnancy) found a trend between sleep problems (assessed using the Sleep Disturbance Scale for Children) and continued use of cannabis during pregnancy relative to no exposure and cannabis use before knowledge of pregnancy (117). Other studies analyzing ABCD data also found significant associations between PCE and several sleep problem scales (121) and that, relative to unexposed offspring, exposed offspring did not benefit from increased sleep in terms of decreased internalizing (mood) problems (122). It should be noted that mothers with cannabis use before and after knowledge of pregnancy were pooled in the Winiger and Hewitt, and Spechler et al., ABCD studies, and thus the amount, duration, and frequency of cannabis exposure over the duration of the pregnancy was not meaningfully assessed, and there is a strong possibility of unmeasured or residual confounding.

Substance use

Risk of cannabis use later in life appears to be elevated following PCE. In adolescent and young adults from the OPPS cohort, PCE was associated with both an increased risk of using cannabis and cigarettes, and an increased risk of daily cigarette smoking (123). Moreover, the risk of subsequent cannabis use following PCE was much higher for male offspring relative to females. In the MHPCD cohort, PCE was predictive of early onset cannabis use (EOCU, before the age of 15) and frequency of use at the 14-year follow up (124). Although PCE was not directly associated with cannabis use disorder (CUD) during young adulthood (i.e., 22-years-of-age) in the MHPCD population, path analysis incorporating longitudinal cohort data was used to examine potential pathways between PCE and CUD (125). Two indirect paths were found; one path led from PCE to CUD through EOCU while another led from PCE to CUD through depression symptoms at age 10 and EOCU.

Summary

Cognitive deficits associated with PCE do not appear to be pervasive across human longitudinal cohorts and seem to appear during specific developmental periods. Relative to other cohorts, the higher risk MHPCD cohort with light to moderate use of cannabis and other substances during pregnancy had more cognitive issues related to PCE exposure. These deficits appeared early in development (prior to age 10) and were more consistently detected during this time. However, it is not possible to assess whether the higher burden of early cognitive deficits associated with PCE in the MHPCD cohort was associated with higher *in utero* cannabinoid exposure or other socioeconomic or environmental factors. Patterns of maternal cannabis use before and during pregnancy were quantified based on self-report in the MHPCD, however associations between use patterns and cognitive outcomes were not always linear. It is not clear whether these results reflect statistical issues or developmentally sensitive periods.

In contrast, attention/impulsivity deficits were more consistently associated with PCE across cohorts and developmental stages. Deficits in attention and impulsivity were often detected during development in the OPPS and MHPCD cohorts and were reported at 18-month-of-age in the GenR study, and during preadolescence (i.e., ages 9–12) in the ABCD cohort. Although associations here are correlative, the combined results across cohorts suggest that PCE may impact brain structures and functions governing attentional processes and impulsivity. Another shared finding across the two most comprehensive longitudinal studies yet completed (e.g., OPPS, MHCDP) was the association between PCE and increased risk of substance use later in life. It will be useful to evaluate offspring substance use patterns in ongoing longitudinal cohorts to see if these associations hold across different study populations, covariates, risk levels, and maternal exposures.

Other findings from human longitudinal cohorts are less clear and point to the need for additional insight and research. For example, it is unclear how PCE influences brain structural changes and sleep during development, largely owing to a lack of data across study populations. Evidence for a role of fetal exposure to cannabis and alterations in aggression, depression, and psychosis appear mixed. Associations between PCE and psychosis and/or externalizing/internalizing traits were detected at different developmental stages across cohorts. However, interpretation of these findings is often complicated because environment, family, and genetic predisposition appear to influence these outcomes as much, or even greater than PCE. Future mechanistic studies in preclinical models and advanced statistical modeling in longitudinal cohorts may be able to dissect complex interactions between PCE, environment, genetics, and their combined influence on behavior.

Support from preclinical studies

Unlike clinical cohorts, preclinical studies have tight control over cannabinoid composition, dose, duration, and

TABLE 3 Litter outcomes following cannabis or THC exposure in preclinical models.

Citation	Model	Maternal exposure period	Dose	Vehicle	Controls	Birth outcome
(128)	CR CD1[M]	G6 through G15, daily	THC (5,15, 50, or 150 mg/kg), oral (gavage)	Sesame oil		No effect on maternal weight gain. No effect on prenatal mortality, fetal weight, or gross morphology.
(129)	C3H/HeJ[M]	Exp1: G0 through birth Exp2: PND21 (mother) through birth	CE (40% THC, 45% CBD) at a dose of 20 mg/kg THC, oral	Olive oil		Exp1: Acute CE resulted in sedation and a suppression of sexual activity but mating still occurred. *Mean gestational length increased by 1 day.* No difference in birth weight. Some loss of pups after birth from unknown causes or cannibalization. Exp2: Decrease in social and sexual behavior but mating still occurred. *Mean gestational length increased by 1 day.* No difference in birth weight. Some loss of pups after birth from unknown causes or cannibalization.
(126)	Swiss-Webster[M]	G6 through birth, daily	THC (25 or 50 mg/kg), s.c	Sesame oil	Pair-feeding and pair-watering; statistical unit is litter	*Reduced litter size and weight at birth. Dose effect on birth weight.* No group differences in maternal weight gain.
(127)	Balb/C[M]	G5.5 through G17.5, daily	Cannabis cigarette (0.3% THC), inhalation	NA	Urine metabolite analysis	No difference in maternal weight gain. No difference in implantation, litter size, fetal growth, or fetal mortality. *Reduction in fetal weight and higher number of males relative to females associated with cannabis treatment. Decrease in fetal-to-placental weight ratio for males. Reduction in fetal lung, brain, thymus, and liver associated with cannabis treatment.*
(140)	CR Albino[R]	Exp1: E14 through PND21 Exp2: E15 through PND21 Exp3: E6 through E15	Exp1: THC or CME (0.5, 1.5, 5.0 mg/kg), oral Exp2: THC or CME (0.5, 1.5, 5.0 mg/kg), oral Exp3: THC and CE (5, 15, 50 mg/kg), oral	Sesame oil	Cross-fostering (Exp2)	Exp1: Tolerance observed after 3–5 days for all treatment groups along with initial and *transient reduction in maternal weight gain at the 5 mg/kg dose.* No impact on gestational length, fetal mortality, fertility, litter size. No difference in pup body weight or sex ratio at weaning. Exp2: Higher mortality at 5 mg/kg THC. Differences in weight and sex ratios at weaning that were not dose dependent. Exp3: *THC and CME treatment groups showed reduced maternal weight gain.* No fetal abnormalities associated with THC or CME.
(139)	H Wistar[R]	G15 through PND9, daily	THC (2.5–5 mg/kg), oral (buccopharyngeal cannula)	Sesame oil		No change in maternal weight gain. No difference in gestational length, litter size, pup weight gain, or postnatal mortality.
(130)	BSF Long Evans[R]	G3 through birth, daily	CE (10 or 150 mg/kg), oral	Olive oil	Pair feeding	*CE reduced maternal food and water consumption and weight gain. Lower birth weight for 150 mg/kg CE dose.* No change

(Continued on following page)

TABLE 3 (*Continued*) Litter outcomes following cannabis or THC exposure in preclinical models.

Citation	Model	Maternal exposure period	Dose	Vehicle	Controls	Birth outcome
						in litter size or pup mortality at birth. Postnatal mortality and neonatal weight both increases in the CE group at weaning (21 days) and females had still not caught up with controls by 11 weeks of age.
(131)	HLA Wistar^{R}	G2 through G22, daily	THC (15 or 30 mg/kg), oral	Sesame oil	Pair fed (food and water); statistical unit is litter	*Lower initial (i.e., first two doses of 30 mg/kg THC) food and water intake. Less weight gain in THC and pair fed groups relative to ad libitum and naïve controls.* No difference in implantation sites, resorptions, perinatal mortality, litter size, or sex ratio. *Positive linear relationship between higher total mortality (i.e., resorptions + perinatal mortality) and pair fed and THC treatment. Lower male birth weight in pair fed and THC treatment groups relative to controls. Lower female birth weight in THC treatment group relative to controls.*
(141)	H Long Evans^{R}	G1 through G22, x2 daily + PND 2 through 10, x2 daily	THC (2 mg/kg), s.c	Ethanol, Tween80, and 0.9% saline (1: 1:18)	Food intake recorded	No difference in maternal weight gain. No difference in gestational length. No difference in weight on PND2.
(132)	CR Sprague-Dawley^{R}	G6 though G15, daily	THC (25, 50, or 100 mg/kg), s.c	Propylene glycol	NA	Lower maternal weight gain (not dose dependent). *Decrease in E20 fetal weight at the 50 mg/kg dose.*
(135)	CR Wistar^{R}	G6.5 through G22, daily	THC (3 mg/kg), i.p	1:18 cremophor: saline	Statistical unit is litter; food intake recorded	No difference in maternal weight gain or food intake. No change in litter outcomes (*i.e.*, gestational length, litter size). *Fetal growth restriction: Decreased liver to body weight ratio at birth.*
(133, 134)	CR Wistar^{R}	G6 through G22, daily	THC (3 mg/kg), i.p	1:18 cremophor: saline	Statistical unit is litter	No change in maternal food intake or weight gain. No difference in litter size or gestational length. *Fetal growth restriction: Symmetrical fetal growth restriction (i.e., reduction in weight and length); THC exposure associated with decreased birth weight and heart-to-body weight ratio, liver-to-body-weight, and brain-to-body-weight ratio. Altered phenotype in E19.5 placenta* (*i.e.*, reduced fetal to placental weight ratio, increased labyrinth layer area and reduced expression of labyrinth progenitors, vascular defects).
(136, 137)	Wistar^{R}	G1 through G19	CE (THC ~3.3 mg), smoke inhalation (filter-tipped cigarette)	NA	Cross-fostering; statistical unit is litter	*Lower birth weight.* No difference in litter size, gestational length, sex ratio, live births, and morphology at birth

(Continued on following page)

TABLE 3 (*Continued*) Litter outcomes following cannabis or THC exposure in preclinical models.

Citation	Model	Maternal exposure period	Dose	Vehicle	Controls	Birth outcome
(142, 143)	CR Sprague-Dawley[R]	G5 through G20, daily	THC (100 mg/mL at 2 L/min airflow) inhalation (e-cigarette)	Propylene glycol	THC metabolites measured during pregnancy; food and water intake recorded	No difference in maternal weight gain or food and water intake. No change in litter outcomes (*i.e.*, gestational length, litter size, sex ratio, or birth weight).
(138)	S Long Evans[R]	G1 through PND2, x2 daily	CE (400 mg/mL) inhalation (e-cigarette)	80% propylene glycol/20% vegetable glycerol	Cross-fostering (most litters contained pups from all conditions)	No difference in litter size. *On PND 6, 10 and 13 air exposed offspring weighed more than CE and vehicle exposed offspring and CE exposed pups weighed more than vehicle exposed pups.*

M, mice; R, rats; BLO, Biobreeding Laboratories Ottawa; BSF, Blue Spruce Farms; CR, Charles River; H, Harlan; HLA, Hilltop Lab Animals; J, The Jackson Laboratory; S, Simonsen Laboratories; s.c., subcutaneous; i.p., intraperitoneal; Exp, experiment; italicized text indicates significant maternal effects; italicized text indicates significant litter outcomes.

frequency of prenatal exposure and can leverage randomized experimental designs to determine causal associations between exposure and experimental outcomes. As summarized below (Tables 3–5), there is some evidence (based on rodent models) that *in utero* exposure to cannabis or its major psychoactive constituent, THC, is associated with adverse birth outcomes and long lasting developmental and behavioral deficits.

Animal models complement clinical studies and provide insight into the range of aberrant offspring outcomes and potential underlying molecular mechanisms following different levels of PCE. For animal models to provide valuable information, one of the important considerations is the comparison between the timing of exposure in the model organism and determining the comparable time in human brain development. Many studies have undertaken these comparisons and identified when specific developmental events, such as the beginning of cortical neogenesis, happen across species. It is now well-established that the first trimester, in terms of brain development, extends until embryonic day (E) 11–13 in mice and E12-15 in rats while the third trimester equivalent happens entirely postnatally in both species. Thus, a host of exposure paradigms have been used that encompass varying epochs in brain development.

To maximize translational relevance in the following review of preclinical studies, this review only reports findings where cannabis/cannabinoid exposure approximates the level of exposure that could reasonably be expected in pregnant women (e.g., smoking cannabis three times per day or less). In addition, only studies that used THC or cannabis extracts (CEs) were included. Similar to the situation in humans, there are several important considerations when interpreting the preclinical findings reported below. First, maternal cannabis exposure has the potential to influence both maternal nutrition (i.e., involuntary exposure may cause decreased food and water intake) and maternal care. Second, the route of administration used to deliver cannabinoids may influence the rate of THC absorption and fetal exposure. Third, the vehicle used to deliver THC, CEs, or cannabis may contain components that produce maternal or offspring developmental effects. Finally, as mentioned above, the duration of exposure and developmental stage is not uniform across preclinical studies. To address some of these caveats we have included information in the tables regarding inclusion of any experimental controls to address maternal cannabis exposure, vehicle composition, duration of exposure, dose, and route of administration. Note that some controls, such as cross fostering, may have complicated consequences on maternal care and pup development.

Birth outcomes associated with PCE in rodents

There is abundant heterogeneity in the experimental design of preclinical PCE studies (Table 3). Despite this variability, a reduction in birth weight associated with PCE is consistently reported across both mouse and rat studies and this result is also commonly replicated in human studies (Table 1).

In mice there was a marginal relationship between THC dose and litter outcomes. In Swiss Webster and Balb/C mice, daily administration of 25–50 mg/kg THC (*s.c.*) or daily exposure to cannabis cigarettes (5 min exposure to 200 mg cannabis cigarette with 0.3% THC content, inhalation) resulted in a reduction of fetal/birth weight (126, 127). However, daily oral administration of THC or cannabis extracts from 5 to 150 mg/kg had no effect on birth weight in CD1 and C3H/HeJ mice (128, 129). Moreover, litter size, sex ratio, fetal mortality, gestational length, and gross morphology were generally unaffected by PCE at the range of doses reported (Table 3). The exceptions being that a daily oral dose of 20 mg/kg THC slightly increased gestational length in C3H/HeJ mice (129) and exposure to cannabis

TABLE 4 Behavioral changes following developmental THC exposure measured in rodents.

Citation	Species/Strain	THC dose & route	Time of admin	Behavior paradigm	Age examined	Outcomes	Sex effects
(164)	Mice: CB1 Conditional KO	3 mg/kg IP inj.	E10–E17	Spatial Memory	P60	Males Impaired Memory	Males
				Object Recognition	P60	No Diff.	No Diff.
(152)	Rat: Wistar	5 mg/kg SC inj.	E5–E20	Social interaction	Adult	Males Decreased Interaction	Males
				Anxiety-like Behavior	Adult	No Diff.	Not Tested
				Cognition	Adult	No Diff.	Not Tested
(33)	Mice: Serotonin Reporter	5 mg/kg IP inj.	E10–E18	Social Interaction	Adult	Decreased Interaction	Not Tested
(172)	Mice: CB1R KO	3 mg/kg IP inj.	E12–E16	Fine Motor Tests	10 weeks	Decreased Fine Motor	Not Tested
(173)	Rats: Sprague Dawley	20 ng/ml Vape	E5–E20	Motor Development	P12–P20	Delayed Capability	No Diff.
				Motor Coordination	P30–32	Poorer	No Diff.
				Activity Level	P31–34	More Activity	Males
				Anxiety-Like Behavior (Center vs. Edge)	P31–34	No Diff.	Males
(174)	Rats: Sprague Dawley	100 mg/ml Vape	E5–E20	Sensori-Motor Development	P12–P20	No Diff.	No Diff.
				Motor Coordination	P30–32	No Diff.	No Diff.
(154)	Rats: Long Evans	0.15 mg/kg IV inj.	E5–P2	Rewarding Behavior	P62	Increased Motivation	Study Includes Males Only
				Depression-like Behavior	P62	Enhanced Depression-like Phenotypes	Study Includes Males Only
(153)	Rats: Sprague Dawley	2 mg/kg SC inj.	E5–20	Locomotor Activity	P28–40	No Diff.	Study Includes Females Only
				Risk Taking	P28–40	No Diff.	Study Includes Females Only
				Anxiety-like Behavior	P28–40	No Diff.	Study Includes Females Only
				Social Behavior	P28–40	No Diff.	Study Includes Females Only
				Anhedonia	P28–40	No Diff.	Study Includes Females Only
				Emotional Memory	P28–40	No Diff.	Study Includes Females Only
(160)	Rats: Sprague Dawley	5 mg/kg Oral	E15–P9	Neonatal Reflexes	P1–P11	Delay in Reflex Development	Not Tested
				Locomotor Activity	P100	No Diff.	Not Tested
				Social Behavior	P100	Decreased Interactions	Not Tested
				Cognitive Behavior	P100	Altered	Not Tested
(156)	Rats: Sprague Dawley	2 mg/kg SC inj.	E5-E20	Depression-like Behavior	Prepuberty	Altered	Study Includes Males Only
				Sensorimotor Gating	Prepuberty	Altered	Study Includes Males Only
(138)	Rats: Long-Evans	2 doses: 400 mg/ml 50 mg/ml Vape	Prior to mating up to P10	Ultrasonic Vocalization	P6, P10, P13	Increased at P6	No Diff.
				Social Play Behavior	P26	Decreased	Males (Some Measures)
				Anxiety-like Behavior	P27	No Diff.	No Diff.
				Adult Anxiety-like Behavior	P73	Increased Anxiety-like Behavior	No Diff.
				Behavioral Flexibility	Adult	Increased Errors (High Dose Only)	No Diff.
(145)	Rats: Wistar	2 mg/kg SC inj.	E5–E20	Locomotor Activity	P25 and beyond	Increased	Not Tested
				Learning & Memory	P25 & Beyond	No Effect	Not Tested

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TABLE 4 (*Continued*) Behavioral changes following developmental THC exposure measured in rodents.

Citation	Species/Strain	THC dose & route	Time of admin	Behavior paradigm	Age examined	Outcomes	Sex effects
				Aversive Limbic Memory	P25 & Beyond	Impaired Performance	Not Tested
				Motivation For Alcohol (Operant)	P25 & Beyond	Increased Motivation	Not Tested
				Nociception	P25 & Beyond	No Diff.	Not Tested
(151)	Rats: RjHan: Wistar	2 mg/kg SC inj.	P1–P10	Ultrasonic Vocalization	P9, P15	Altered (Both Ages)	Not Tested
				Homing Behavior	P10, P13	No Diff.	Not Tested
(162)	Rats: RjHan: Wistar	2 mg/kg SC inj.	P1–P10	Locomotor Activity	Adult	No Diff.	No diff.
				Social Interaction	Adult	Enhanced Interaction, Diminished Social Memory	No Diff.
				Memory	Adult	No Diff.	No Diff.
(161)	Rats: Sprague Dawley	5 mg/kg Oral	E15–P9	Social Interaction	P180	Decreased interaction	Study Includes Males Only
				Memory	P180	Impaired (Decreased Retention)	Study Includes Males Only
(56)	Rats: Sprague Dawley	0.15 mg/kg IV inj.	E1–E21	Passive Avoidance	P22	Impaired retention	No Diff.
				Active Avoidance	P45	Impaired Reversal	Males
				Attention	P60	Impaired	No Diff.
				Amphetamine Challenge	P60	Dampened Response	No Diff.
(139)	Rats: Wistar	2.5 or 5 mg/kg Orally through Cannula	E15–P9	Ultrasonic Vocalization	P12	Increased (High Dose)	Not tested
				Social Interaction	P35	Decreased Interactions	Not Tested
				Anxiety-like Behavior	P80	Increased Anxiety-like Behavior	Not Tested
(141)	Rats: Long Evans	2 mg/kg SC inj.	E1–E22, P2-10	Activity level	P90	No Diff.	Study Includes Males Only
				Anxiety-like Behavior	P90	Increased Anxiety-like Behavior	Study Includes Males Only
				Social Interaction	P90	Increased Interaction	Study Includes Males Only
				Depression-like Behavrior	P90	No Diff.	Study Includes Males Only
(169)	Rats: Wistar	5 mg/kg Orally through cannula	E15–P9	Inhibitory avoidance	P80	Increased Avoidance	Study Includes Males Only
				Social Discrimination	P80	Impaired Discrimination	Study Includes Males Only
(53)	Rats: Long Evans	0.15 mg/kg IV inj.	E5–P2	Heroin Self-administration	P62	Increased Self-Administration	Not Tested
				Stress-Induced Heroin Administration	P62	Enhanced Administration	Not Tested
				Heroin-Induced Locomotor Activation	P62	Decreased Activity	Not Tested
(148)	Rats: Wistar	5 mg/kg IP inj.	P4–P14	Heroin-induced place conditioning	P56	Increased Preference	Not tested
				Heroin-Induced Locomotor Activation	P56	Effects Observed at Low Dose of Heroin	Not Tested
(175)	Rats: Wistar	5 mg/kg SC inj.	P4–P14	Spatial Discrimination	P56	No Diff.	Study Includes Males Only
				Delayed Alternation	P56	Delayed Acquisition	Study Includes Males Only
(150)	Rats: Wistar	5 mg/kg Oral	E5–P24	Morphine self-administration	P70	No Diff.	No THC -induced diff.
(146)	Rats: Wistar	1 or 5 mg/kg	E5–P24	Morphine conditioned place preference	P70	Enhanced Preference	Males (Both Doses) Females (Low Dose)

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TABLE 4 (*Continued*) Behavioral changes following developmental THC exposure measured in rodents.

Citation	Species/Strain	THC dose & route	Time of admin	Behavior paradigm	Age examined	Outcomes	Sex effects
				Anxiety-like Behaviors	P70	Less Anxiety	Males
(52)	Rats: Wistar	5 mg/kg Oral	E5–P24	Morphine self-administration	P70	Enhanced Administration	Females
(147)	Rats: Wistar	5 mg/kg Oral	E5–P24	Open Field Behavior	P70	Increase in Behaviors	No Diff.
				Locomotor Activity	P70	Increased Activity	Females
				Morphine Place Preference	P70	Enhanced	No Diff.
(149)	Rats: Wistar	5 mg/kg Oral	E5–P24	Opioid Withdrawal After Naloxone Challenge	P24	Increased Symptoms	Males
				Morphine-Induced Analgesia	P70	Increased Analgesia	Males
(155)	Rats: Wistar	5 mg/kg Oral	E5–P24	Development of motor behaviors over time	P15, P20, P30, P40, P70	Altered on Specific Days	Sexually Dimorphic Based on Day
				Stress-Induced Motor Behaviors	P70	Altered	Females
(144)	Rats: Fischer-344	10 mg/kg Inj.	P4, P6, P8	Stress-induced tail withdrawal	P130	Increased Withdrawal Latency	Not tested

cigarette smoke altered litter sex ratios in favor of males and contributed to fetal growth restrictions in male treated Balb/C mice (127).

In rats, perhaps owing to a greater number of studies, there was an apparent linear relationship between THC dose and litter outcomes. In Long Evans, Wistar, and Sprague Dawley rats, lower birth weight was associated with daily *in utero* exposure to 15, 30, or 150 mg/kg (oral) cannabis extracts (130, 131), 50 mg/kg (s.c.) THC (132), 3 mg/kg (i.p.) THC (133–135), or smoke from cannabis extracts (400 mg/mL) (130, 136–140). In contrast, no effect of PCE on birth weight was observed following daily *in utero* exposure to 0.5–10 mg/kg (oral) THC or CE (130, 139, 140), 2 mg/kg (s.c.) THC (141), or smoke from CEs (100 mg/mL) (142, 143). In addition, placental alterations were associated with daily *i.p.* injections of THC (3 mg/kg) (134). Litter outcomes did not appear to be strongly influenced by differences in maternal food and water intake or weight gain following cannabinoid exposure (Table 3).

Taken together, preclinical studies in both mice and rats support the major clinical finding of reduced birth weight and fetal growth restriction associated with PCE (Table 1). Because cannabinoid composition and dose can be well controlled in preclinical studies, an additional finding with relevance to human studies is that there is a strong negative effect of THC dose on adverse outcomes. In preclinical rat studies this holds true regardless of the route of administration. For example, exposure to THC above 10 mg/kg (oral), 2 mg/kg (s.c.), or 100 mg/mL (inhalation) in rats resulted in adverse birth outcomes. There are a smaller number of studies in mice and the impact of route of administration and THC dose is less clear, perhaps due to genetic differences among strains or experimental paradigms. More work is needed in mice to evaluate the impact of THC dose and route of administration on litter outcomes. Taken together, preclinical studies in rodents suggest that exposure to higher levels of THC increases the risk of adverse birth outcomes.

Exogenous cannabinoid exposure has the potential to disrupt reproductive processes and alter mating behavior. For this reason, many preclinical studies delay cannabinoid treatment until G5 or G6 in the hopes of increasing the odds of pregnancy. Several studies varied maternal cannabinoid exposure to quantify changes in fertility, productive mating, and implantation (129, 131). However, there appeared to be little effect of low to moderate cannabinoid exposure (15–30 mg/kg, oral) on these outcomes. This is an important consideration as most human mothers consume cannabis prior to pregnancy and during the first trimester with use tapering off in later trimesters (4). Use throughout pregnancy is less common and may be associated with higher risk pregnancies and other maternal characteristics that could influence birth outcomes and later

child development. In rodents, the third trimester equivalent occurs outside of the mother's body. Many of the studies in Table 3 included daily exposure to cannabinoids from G6 through G22, which corresponds to the first and second trimesters in humans. In the rodent studies, daily first and second trimester PCE was often sufficient to cause adverse litter outcomes (i.e., low birth weight).

Long-lasting impact of PCE on behavioral and molecular outcomes in rodents

Preclinical studies in rodents have provided additional insight into the effects of PCE on a wide range of traits. Examination of the effects of cannabis on brain development began in the 1970s and 1980s [e.g., (144)]. These studies gave the first clues on the types of effects that cannabis had on the developing brain and some studies showed that similar types of behavioral effects were observed in animal models and human populations. With the legalization of cannabis for recreational and/or medical use in many parts of the US, there has been a resurgence in research on its effects on brain development. Similar to what is observed in human populations, the results are not always consistent across preclinical studies. This is likely due to variations in a range of experimental parameters including differences in doses, time of exposure, time of evaluation of the behavioral phenotype, and testing parameters. However, even with these differences, it is clear that a number of different effects have been observed in animals following PCE and the findings are summarized below.

Substance use

One of the most consistent findings is that prenatal exposure to THC alters the behavioral response to other drugs of abuse, notably opioids, taken later in life. In these experiments, animals are exposed to THC during prenatal or early postnatal periods followed by exposure to a different drug of abuse during adolescence or adulthood [e.g., (52, 53, 56, 145–149)]. These studies have demonstrated that PCE can influence the rewarding/reinforcing properties and behavioral or physiological responses to other drugs later in life (Table 4). Only a single study failed to show differences between animals with PCE and unexposed controls (150). These results suggest long lasting effects of PCE that may enhance the propensity for substance use disorders later in life.

Externalizing/internalizing behavioral problems

Emotional reactivity has been examined in a host of behavioral paradigms and over varying ages. Several studies have examined ultrasonic vocalizations in pre-weanling pups, which are defined as cries from the pup to the dam and interpreted as a sign of distress. The results have shown that THC-exposed pups show altered vocalizations, at least at certain ages, supporting the hypothesis that developmental THC exposure can alter early emotional responding (138, 139, 151). Numerous studies have examined other responses later in life with mixed results. For example, anxiety-like phenotypes have

TABLE 5 Behavioral outcomes following prenatal polysubstance exposure.

Author	Co-exposure	Exposure parameters	Behavior tested	Thc only effect	Interaction	Sex effect
(173)	THC + Ethanol	GD 5–20 (rats) THC (100 mg/ml; 30 min vapor Inhalation at airflow rate of 2L/min in e-cigarette tank); Ethanol (BAC = 150 mg/dl; vapor inhalation)	Motor Development	Delayed Capability	Enhanced	No
			Motor Coordination	Poorer	No	No
			Activity Level	More Activity	Increased	Males
			Anxiety-like Behavior (Center)	No	Increased Time in Center	Males
(174)	THC + Nicotine	GD 5–20 (rats); THC: 100 mg/ml; Nicotine: 36 mg/ml; 40 min vapor inhalation at airflow rate of 2L/min in e-cigarette tank	Sensori-Motor Development	No	Delayed Success	No
			Motor Coordination	No	Lower Success Rate	Females
(176)	THC + Nicotine	Through Gestation (rats); THC (Oral, Edible); Nicotine (Vapor Inhalation)	Sensori-Motor Gating	No	Deficits	Males
			Memory	Yes	Males (short-term memory deficit with THC + Nicotine)	Males (short-term memory deficit with THC or THC + Nicotine) Females (short-term memory deficit with THC alone)
			Anxiety-Like Behaviors	Increased Anxiety-Like Behaviors	No	Males

been examined and results have shown increased anxiety-like behavior (138, 139, 141), decreased anxiety-like behavior (146), and no difference from controls (152, 153). Stress reactivity and depression-like phenotypes are not as well-studied. Prenatal THC exposure has been shown to alter both phenotypes in some studies (144, 154–156), while others have shown no significant effects (141, 153).

Psychosis

In recent years, it has been proposed that PCE may be a risk factor for psychosis later in life as part of a "two-hit" model [see (157) for review]. The proposed mechanisms behind this model state that prenatal exposure to THC alters the cannabinoid system during development. The second hit happens when an additional environmental exposure occurs, such as stress or THC exposure later in life, that further disrupts the endocannabinoid system ultimately leading to psychosis. Psychosis and Schizophrenia are uniquely human disorders but some aspects of the disease (i.e., endophenotypes) can be studied in rodents. One such endophenotype is altered sensory gating evaluated using the pre-pulse inhibition paradigm or PPI [see (158) for review]. In one of the first studies to evaluate this (159), rats were exposed to THC *in utero* and later during adolescence. The results showed altered sensorimotor gating effects in animals exposed to THC both *in utero* and during adolescence compared to those exposed only *in utero,* only at adolescence, or unexposed controls. Interestingly, the results were observed in males but not in females suggesting some sex-specificity of the effects. These types of sensorimotor gating effects have been replicated in further experiments [e.g., (156)] suggesting the importance of further evaluating this relationship.

Social interactions

Mice and rats are highly social animals and have often been evaluated for the frequency and type of social interactions. Social behavior has been evaluated across multiple paradigms. For many of these studies, prenatal THC exposure has been shown to alter social interactions (33, 138,141,152,160,161,162). In contrast, a study by Traccis and colleagues (153) failed to show an effect of prenatal THC on social behaviors.

Brain regions and signaling pathways

One advantage of preclinical studies is the ability to dissect the underlying mechanisms associated with changes in behavior following PCE. Although a detailed analysis of current studies is beyond the scope of this review, we touch on a few findings that highlight the utility of rodent models in dissecting the functional implications of changes in brain signaling systems following PCE. Long-lasting alterations in brain reward regions (i.e., the mesolimbic dopamine system including the ventral tegmental area and nucleus accumbens) have been reported following PCE. Changes to this system may mediate enhanced propensity for drug seeking/reinforcing behavior in rodents and risk of substance use and abuse in humans. In rats, PCE is associated with altered dopaminergic function in the ventral tegmental area (159, 163) and decreased expression of dopamine receptor genes (i.e., dopamine receptor D2) in the nucleus accumbens (48). Decreased levels of dopamine receptor D2 mRNA in nucleus accumbens following PCE have also been observed in human fetal tissue (48).

Alterations in cortical and hippocampal regions following PCE could mediate changes in cognition, memory, attention, and impulsivity. Changes in the number or function of hippocampal inhibitory neurons (33, 164, 165) and alterations in both inhibitory hippocampal GABAergic transmission (166) and excitatory hippocampal glutamatergic neurotransmission (167) have been reported following PCE in rats. Prenatal or perinatal exposure to THC or cannabinoid agonists have also been associated with changes in cortical synaptic plasticity and excitatory glutamatergic signaling (51, 168, 169). These are just a few of the many rodent studies that are beginning to quantifying precise molecular and functional perturbations to brain signaling pathways following PCE [for review see (170, 171)].

Sex, genetic background, and other variables

In addition to confirming results seen in human populations, animal models have also extended these findings particularly by evaluating other variables. One of the most notable examples is the effects of sex. As seen in Table 4, several studies have shown sex-specific effects, although there are a number of studies in which only one sex was evaluated leaving open the question of sex-specific effects. This is clearly an issue that warrants further study in human populations which will likely be facilitated by characterization of additional populations. Surprisingly, the role of genetics in modulating the potential teratogenic effects of THC have been minimally explored, at least as it relates to behavioral effects. Most of the experiments have focused on examination of specific components of the THC pathway, such as the CB1 receptor, using knock out mice [e.g., (164, 172)]. This is also an issue that warrants additional study. Moreover, the role of other variables, such as nutrition or exposure to stress, need additional investigation.

Prenatal polysubstance exposure

Preclinical models are also invaluable for separating the effects of THC from that of other drugs. The rate of maternal polydrug use during pregnancy is high making it difficult to assess whether effects are due to THC exposure or to exposure to other substances of abuse. As discussed previously, data from

preclinical studies demonstrates the potential teratogenic effects of THC on a range of outcomes. However, there can also be interactions among drugs, either in positive or negative directions, and the nature of these interactions can be evaluated in preclinical models.

One of the most commonly co-abused substances is ethanol. Ethanol has been shown to be highly teratogenic with widespread effects in many CNS regions and on many developmental processes. Behaviorally, the effects of ethanol exposure have been shown to have some similarities with those of THC including effects on anxiety- and depression-like phenotypes. As shown in Table 5, studies are beginning to evaluate co-exposure to both ethanol and THC (173). While the results demonstrate that each substance alone can cause behavioral alterations, there is also evidence for an interaction of the two drugs suggesting that the co-exposure can worsen the teratogenic effects of each alone.

A second drug with high rates of co-exposure is nicotine either through traditional cigarettes or through e-cigarettes. While limited in nature, studies are beginning to evaluate this co-exposure as well (Table 5). Similar to what was observed for ethanol, different effects of either substance alone or after combined exposure have been found (174, 176). Pronounced sex effects were also observed, suggesting that sex may be a variable in the response to the co-exposure of these two drugs of abuse. Additional work is needed to further expand this interaction.

Summary

Preclinical rodent models are beginning to quantify the teratogenic profile of cannabis and THC on offspring outcomes following PCE. In these studies, outcomes are specifically associated with *in utero* cannabinoid exposure rather than exposure to other substances of abuse or uncontrolled environmental factors. However, there are limitations to these studies. These include heterogeneity in experimental design (e.g., variation in methods of exposure, dose, cannabinoid, timing of exposure) and phenotypic outcomes measured. Further, because the level of brain development in a newborn rodent differs from that in a newborn human, translating across species can be difficult. Even with these limitations, there are consistent findings across studies on a range of phenotypes including lower weight at birth, altered emotionality, and altered responses to and enhanced propensity to consume other drugs of abuse later in life. Significant associations between PCE and other behavioral deficits have been reported, although the results are not always consistent across studies. Further work is needed to resolve these differences. Moreover, there are numerous issues that are difficult to address in clinical studies that still can be explored further using rodent PCE models. First, it is still unclear whether there are levels of maternal cannabis use that might be associated with less risk to child development. It is also unclear how cannabis composition, especially potency, might influence the risk of adverse birth outcomes or child developmental problems later in life. Second, the contribution of genetic background and/or environmental variables to the susceptibility of teratogenic effects following PCE remain understudied and unclear. Finally, we still know relatively little about the underlying molecular mechanisms and changes to brain structure and function impacted by PCE. Preclinical models are beginning to address the underlying molecular changes associated with PCE and have implicated several neurotransmitter systems, brain regions, and cell types of interest but more work is needed to evaluate structural and functional changes following PCE. This knowledge will be needed to design and evaluate interventions that ameliorate the teratogenic effects of cannabis, particularly as they relate to psychosis risk.

Discussion

Maternal cannabis use and cannabis potency are both increasing despite the possible adverse and long-term consequences of PCE on child development. However, research to date has yet to establish clear links between adverse offspring outcomes and the frequency, duration, and types of maternal cannabis exposure. Longitudinal studies in humans are important for tracking potential long-lasting outcomes associated with prenatal cannabis exposure. However, all studies to date suffer from small sample sizes, potential confounding factors, outcome measures that differ between studies, and the inability to quantify cannabinoid exposure during pregnancy. Moreover, the prospective design and a lack of randomized conditions in human study cohorts precludes causal inference between *in utero* cannabis exposure and later health and behavioral outcome measures.

Despite these limitations, clinical cohorts and longitudinal studies are beginning to illuminate some of the potential consequences of PCE on offspring outcomes. These include adverse birth outcomes (e.g., low birth weight, premature delivery, admission to neonatal intensive care) and longer lasting behavioral alterations in offspring with PCE (e.g., attention and impulse control deficits, elevated risk of substance use disorders, and a possible increase in psychopathology, aggression, anxiety, and depression). In preclinical models, cannabinoid exposure and environmental conditions can be tightly controlled, and randomized study designs can be applied to infer causality. Moreover, intervening molecular pathways from PCE to phenotypic outcome can be identified as can variables (e.g., exposure level, sex, genotype, environment) that influence outcome severity. Studies leveraging rodents lend support to the hypothesis that human PCE causes a reduction in birth weight and fetal growth restriction, with a longer-term impact

on offspring behavior across multiple domains related to substance use, emotional reactivity, and psychopathology.

Much work is still needed to characterize the full spectrum of teratogenic effects associated with different levels of *in utero* exposure to cannabis and cannabinoids. Moving forward, study designs in rodents will need to better model human patterns of cannabinoid exposures, especially variation in cannabis potency and composition, routes of administration, polysubstance interactions, and maternal pre- and post-conception exposures. Clarification of the range and types of offspring phenotypes impacted by PCE, enhanced translational relevance of phenotypes, and better replication of comparable outcomes across studies will be required for clinical and preclinical studies. Rodent studies may be especially useful to disentangle the role of confounding factors (e.g., sex, genetics, multi-drug interactions, and other environmental variables) that have been difficult to model in human studies where sample sizes can be small relative to the number of potential covariates. Finally, the addition of developmental brain molecular, functional, and structural data has the potential to bridge the gap between PCE and behavioral outcomes across species. Rodent studies are beginning to resolve the role of discrete signaling pathways and brain regions in modulating behavioral outcomes following PCE. However, functional and structural imaging studies of brain development following PCE are lacking in humans and rodents. Ultimately, an important goal of future research is to clearly define developmental processes vulnerable to PCE and provide clinicians and patients with more detailed information about specific risks to the child posed by different levels of maternal cannabis exposure.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Vascular contributions to the neurobiological effects of prenatal alcohol exposure

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Background: Fetal alcohol spectrum disorders (FASD) are often characterized as a cluster of brain-based disabilities. Though cardiovascular effects of prenatal alcohol exposure (PAE) have been documented, the vascular deficits due to PAE are less understood, but may contribute substantially to the severity of neurobehavioral presentation and health outcomes in persons with FASD.

Methods: We conducted a systematic review of research articles curated in PubMed to assess the strength of the research on vascular effects of PAE. 40 pertinent papers were selected, covering studies in both human populations and animal models.

Results: Studies in human populations identified cardiac defects, and defects in vasculature, including increased tortuosity, defects in basement membranes, capillary basal hyperplasia, endarteritis, and disorganized and diminished cerebral vasculature due to PAE. Preclinical studies showed that PAE rapidly and persistently results in vasodilation of large afferent cerebral arteries, but to vasoconstriction of smaller cerebral arteries and microvasculature. Moreover, PAE continues to affect cerebral blood flow into middle-age. Human and animal studies also indicate that ocular vascular parameters may have diagnostic and predictive value. A number of intervening mechanisms were identified, including increased autophagy, inflammation and deficits in mitochondria. Studies in animals identified persistent changes in blood flow and vascular density associated with endocannabinoid, prostacyclin and nitric oxide signaling, as well as calcium mobilization.

Conclusion: Although the brain has been a particular focus of studies on PAE, the cardiovascular system is equally affected. Studies in human populations, though constrained by small sample sizes, did link pathology in major blood vessels and tissue vasculature, including brain vasculature, to PAE. Animal studies highlighted molecular mechanisms that may be useful therapeutic targets. Collectively, these studies suggest that vascular pathology is a possible contributing factor to neurobehavioral and health problems across a lifespan in persons with a diagnosis of FASD. Furthermore, ocular vasculature may serve as a biomarker for neurovascular health in FASD.

KEYWORDS

hypertension, fetal alcohol spectrum disorder, cardiovascular system, vascular pathology, retina

Introduction

Prenatal alcohol exposure (PAE) is well documented to result in a range of adverse physical and neurobehavioral outcomes that are collectively subsumed under the term *Fetal alcohol spectrum disorders* (FASD). Though not by itself an accepted diagnostic term, FASD is a comprehensive umbrella term that includes several diagnostic classifications (1, 2). At the severe end of the FASD continuum, affected individuals may exhibit musculoskeletal and growth deficits, and experience profound craniofacial anomalies, including ocular defects, mid-face hypoplasia and/or cleft palate, profound brain anomalies such as agenesis of the corpus callosum, microencephaly, and intellectual disability (3-8). Evidence for a cluster of presenting symptoms, including mid-face anomalies, growth deficiencies including brain growth deficits and neirobehavioral impairment allows for the diagnosis of *Fetal Alcohol Syndrome* [FAS, for a comprehensive description of diagnostic criteria, see (2)]. However, a majority of persons along the FASD continuum do not exhibit obvious physical anomalies, but rather neurobehavioral deficits including deficits in memory, attention, mathematical and language skills, and decision making processes (9–11). These deficits can have an equally adverse impact on quality of life. These latter, and more prevalent outcomes, are defined by the term *Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure* (ND-PAE) (12, 13), which was incorporated for the first time into the 5th edition of the Diagnostic and Statistical Manual of American Psychiatric Association as an acceptable diagnosis, absent obvious physical anomalies.

PAE is unfortunately very common. Recent studies have investigated levels of phosphatidyl ethanol in blood samples obtained from newborn infants, a unique molecular adduct formed on erythrocyte phospholipid membranes following ethanol exposure. These studies in Texas (14) and West Virginia (15) in the US, and Ontario in Canada (16) documented positivity for this marker in 8%–15% of newborn samples, indicative of substantial rates of third-trimester exposure in these populations. Even higher rates of positivity have been documented in newborns from selected high-risk populations elsewhere (17). Unsurprisingly, estimates of FASD prevalence are also high. For instance, a recent and large prospective case-ascertainment study in four school systems across the US estimated rates of FASD in school-aged children of 1.1%–5%, with a weighted prevalence estimate of 3.1%–9.8% (18). Worldwide, previous estimates have placed the prevalence of FASD at as high as 11.3% in some regions like South Africa, with rates equivalent to North America in many European nations (19). These data collectively point to the likely outsized contribution of PAE to the burden of developmental disability in North America and world-wide, and emphasize the need to understand the etiology of FASD with the goal of early interventions to mitigate the effects of PAE.

The centrality of brain-based disability to the diagnosis of FASD, and particularly ND/PAE, mean that most research has focused on developmental perturbations to neural cells and on the neurobiology of this disability. However, in this review, we focus on the literature implicating the cardiovascular system, and specifically the contribution of the cerebrovascular system in the etiology of FASD. The cerebrovascular system develops during the peak period of neurogenesis (20), and one recent study suggests that cerebral neurogenesis and angiogenesis is molecularly linked, for example, by a microRNA, miR-9 (21). We previously found miR-9 to be inhibited by ethanol in mouse neural stem cells (22), with inhibition in zebrafish resulting in the loss of brain tissue (23). These data suggest that the effects of ethanol on neurogenesis and angiogenesis are mechanistically linked. Conversely, the loss of the endothelial receptor for endothelin-B (ET_B) has been documented to result in microencephaly, i.e., reduced brain size (24), a key feature of severe FASD, emphasizing the interdependency of neural and vascular systems in the growth of the fetal brain. Interestingly, a few studies using functional magnetic resonance imaging to document changes in functional network connectivity in the resting state (25) and following functional activation (26, 27), in children and adult persons with a diagnosis of FASD, have assessed changes in the BOLD (Blood Oxygenation Level Dependent) signal. These are an important collection of papers for the purposes of this review, because changes in the BOLD signal equally implicate vascular adaptation and neuronal circuit activation (28), and dysregulation in the BOLD signal may indicate vascular dysfunction. Here we conducted a systematic PubMed review of papers on FASD and the vascular system, selecting papers that documented vascular deficits in both human populations and animal models, as well as papers that tested underlying mechanisms, to identify the current knowledge state and potential knowledge gaps in the field of vascular effects of PAE.

Methods

We conducted an initial systematic review search in June of 2021 of research articles curated in PubMed to assess the strength of the research on vascular effects of PAE. PubMed was screened for several terms, as listed in Figure 1. After removing all review papers, a total of 191 papers were present using those terms. Of the 191 papers, 24 paper were unavailable to access fully and 99 were unrelated to blood vessels or the cardiovascular system. With 68 papers remaining, non-alcohol related studies were removed, and the remaining 38 studies were then separated by human versus animal models. Subsequent searches conducted until June of 2022 yielded an additional 3 pertinent papers. A total of 40 pertinent papers were selected to be included in this systematic review on the effects of ethanol exposure *in utero* to cardiovascular and neuropsychiatric systems exhibited in both human populations and animal models.

Figure 1: Search Terms
Mothers AND Ethanol
Ethanol AND placental circulation
Ethanol (Major) AND brain OR cerebrum AND blood vessels AND Fetal Alcohol Spectrum Disorders
Ethanol AND fetus AND human
Nicotine OR tobacco AND fetus AND blood vessels
Teratogens AND fetus AND blood vessels
Fetal alcohol spectrum AND blood vessels
Ethanol AND Maternal-fetal relations
Ethanol AND Mother-Child Relations
Fetal alcohol syndrome AND blood vessels

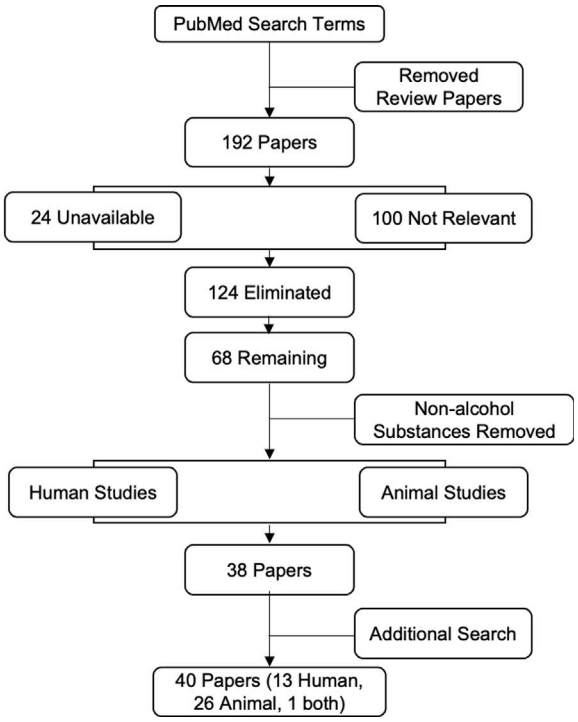

FIGURE 1
Search terms and schematic for analysis.

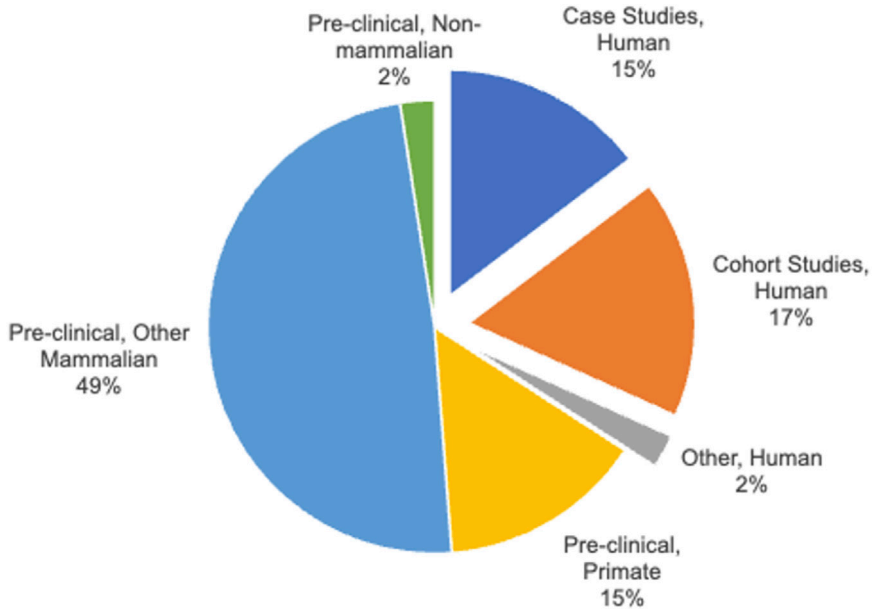

FIGURE 2
Classification of Human and Pre-clinical animal studies. The category, "Pre-clinical, Other Mammal" includes sheep, rats and mice.

Results and discussion

Studies in human populations

A key, but perhaps unsurprising finding from our analyses is that there are very few studies in human populations, particularly on brain vascular effects of PAE (Figure 2). Many of these studies were case reports, based on very small sample sizes. However, a few studies did include both larger samples of persons with a diagnosis of FAS or pFAS, and importantly, well-described reference or comparison group samples. The earliest description of anatomical anomalies associated with FAS specifically included reference to cardiovascular anomalies, including ventricular septal defects (6, 29), and stenosis of major cardiac efferent arteries like the pulmonary artery (30). Another 1979 case report on two infants with a diagnosis of FAS who were surgically treated for cardiac septal defects (31) also noted that the infants exhibited dysplastic pulmonary arteries, and one infant also exhibited aortic insufficiency. A later case report found evidence for stenosis of the descending aorta and the renal arteries in a child diagnosed with FAS (32). These case reports, despite containing very small patient samples, showed that the heart and large arteries could be affected and perhaps, contribute to the pathology of FAS. Furthermore, vascular deficits within the interstitial vasculature of tissues have also been documented in humans diagnosed with FAS. For instance, in an early report in Lancet by Habbick et al (33), analysis of liver biopsies from three children with FAS uncovered sclerosis and other damage associated with central veins in hepatic lobules and other hepatic vasculature. Additional early supporting data on the vulnerability of interstitial vessels comes from a study in placenta samples obtained from control and alcohol-exposed pregnancies (34). Electron microscopy analysis of placental ultrastructure (five cases from both control and alcohol-exposed pregnancies) found evidence for vascular endarteritis and thickened basal lamina of placental blood vessels, suggestive of a potential inflammatory occlusion of blood vessels and restriction of blood flow. The collective assessment from each of these small-scale studies is that vascular damage may not be limited to the large vessels, but instead may be a general feature of tissues and organs as well. Moreover, such damage may limit blood flow to a variety of organs and contribute to impaired organ function. The question is whether such impaired vascular function also occurs in brain.

Cerebral micro-vessel structure in FASD

Our literature review identified two primary studies of brain microvessels, both of which examined postmortem fetal tissues from control cases and cases that met the diagnostic features of FAS or partial FAS (pFAS). The first study, which included 11 FAS/pFAS cases and eight control cases from gestational ages of 19–38 weeks (35), reported that the effects of PAE on brain vasculature were more prominent during later developmental periods. While control fetal brains exhibited a predominantly radial pattern of microvessels that traversed the marginal zone through the cortical plate and intermediate zones of the cerebral cortex, this radial pattern was significantly diminished in older fetuses with features of FAS/pFAS. The lack of observed effects in fetuses at earlier gestational ages suggests that vascular deficits may either result from cumulative PAE, or that the deficits from earlier episodes emerge later in development. More recently, the same research group published a second post-mortem study in four control fetuses and four fetuses with features of FAS/pFAS, ranging from gestational ages of 29–34 weeks (36). Using immunohistochemical staining for the microtubule-associated light chain protein, LC3 (Map1lc3a), the authors found a significant increase in LC3-positive puncta in endothelial cells lining cerebral microvasculature, an outcome that the authors interpreted as increased autophagy. Interestingly, in contrast to the previous study, increased LC3-positive puncta were observed early on, at gestational week 29, in fetuses with characteristics of FAS/pFAS, suggesting that autophagy may precede the loss of radial microvessels. A rigorous component of both studies was that the authors also replicated their findings in murine and cell culture models, suggesting that ethanol is a causal agent in the loss of brain microvessels, and that autophagy, a common stress response to nutrient deprivation, is one mediating factor.

An important caveat in interpreting these studies is their small sample sizes. Furthermore, fetuses affected by high levels of alcohol exposure (characterized as chronic daily alcohol exposure to binge levels) may have also resulted in spontaneous pregnancy termination. Other researchers have documented a link between heavy alcohol exposure during pregnancy and spontaneous pregnancy termination (37, 38). It is therefore possible that the heaviest alcohol exposures, which place pregnancy viability at risk, also compromise fetal vascular development. It remains to be determined if lower levels of prenatal alcohol exposure also compromise fetal brain vascular development in human populations. Another limitation of these studies is that they documented the acute effects of alcohol exposure in the fetus. For assessments in later life, a number of researchers turned to the retina, as a proxy tissue for assessing brain.

Retinal circulation as a marker for cerebrovascular effects of FASD

Like brain, the retina is a central nervous system structure, protected by a structure equivalent to the blood-brain barrier, i.e., the blood-retinal barrier (39). As with the brain, the health of retinal neurons is dependent on an extensive microvascular network. A substantial advantage is that the structure and function of retinal arteries is readily accessible at any stage of postnatal life by standard ophthalmological visualization

TABLE 1 Summary of vascular studies in FASD in human populations.

FASD sample size (comparison group sample size)	Study type	Vascular region	Major findings	References
3 (0)	Case Study	Hepatic	Two cases of children with FAS presented with sclerosis of the central vein and distention, while 1 case displayed hepatic fibrosis and cystic kidney disease	(33)
2 (0)	Case Study	Heart and Lungs	Two infants with FAS presented with hypoplastic pulmonary arteries, congestive heart failure, and ventricular septal defects	(31)
17 (0)	Case Study	Eye	Children with FAS displayed retinal arterial and venous tortuosity, optic nerve hypoplasia, and decreased visual acuity	(40)
5 (0)	Case Study	Placental	There is significant endarteritis, trophoblastic basement membrane thickening, and basal lining hyperplasia in villous capillaries in placenta, in pregnancies that were prenatal alcohol-exposed	(34)
10 (0)	Case Study	Eye	Ten children with FAS or fetal alcohol effects (FAE) presented with ophthalmic defects including vessel tortuosity, optic nerve hypoplasia, cataracts, and visual impairment	(42)
16 (162)	Cohort Study	Eye	FASD associated with increased tortuosity of retinal vessels and decreased vessel branching	(43)
25 (92)	Cohort Study	Eye	117 children diagnosed with optic nerve hypoplasia were further assessed. 25 were also diagnosed with FAS, with some presenting with arterial and venous tortuosity	(44)
1 (0)	Case Study	Abdominal aorta	10-year-old boy with FAS and hypoplastic abdominal aorta presented with severe hypertension and was successfully treated with renal angioplasty	(32)
32 (25)	Cohort Study	Eye	In the sample, 30% showed retinal vessel tortuosity and 25% showed optic disc hypoplasia	(41)
31 (30)	Cohort Study	Brain	fMRI used to measure brain blood flow and blood oxygen levels. Persons with FASD exhibited significantly lower average activation for working memory tasks	(45)
4 (4)	Cohort Study	Brain	PAE increases the number of autophagic vacuoles in brain cortical microvessels	(36)
125 (500)	Cohort Study	Cardiovascular	Children with a diagnosis of FAS or partial FAS were at increased risk for hypertension compared to children in the general population (sampled from the National Health and Nutrition Examination Survey, NHANES)	(46)
37 (35)	Cohort Study	Eye	Observed association between prematurity and increased tortuosity of retinal vessels, strabismus, myopia, and optic nerve anomalies. Non-FASD cases were not further evaluated	(47)

techniques. Not surprisingly, a number of studies on the vascular effects of PAE in human populations have focused on assessing retinal blood vessels. A majority of these studies, including some of the early case reports (40–42), reported increased tortuosity of retinal vessels (see Table 1).

In a larger study evaluating ophthalmologic findings in children with FASD, Gyllencreutz et al (47) assessed a cohort of 30–32 eastern European and Swedish children with FASD, who were longitudinally observed from childhood into adulthood for persisting ophthalmologic effects following PAE. The children had a median age of 7.9 years at the time that a multidisciplinary team diagnosed them with FASD, and evaluated visual acuity, stereoacuity, ocular media, strabismus, refraction, and fundus. At 13–18 years later (with the median age of study participants at 22 years old), the study cohort was reexamined and many of the earlier documented ophthalmologic findings - including astigmatism, defective stereoacuity, heterotropia, and optic nerve hypoplasia - were found to persist into adulthood. In addition, an increased tortuosity of retinal vessels was noted to persist into adulthood. Although none of the children enrolled in this study were born extremely premature, this study did include 12 children with a premature birth history (31–36 weeks of gestation), 16 children with a birth weight of less than 2,500 g, and 12 children who were born small for gestational age (SGA). These are important considerations, because, as we discuss later, prematurity is linked to increased tortuosity of retinal vessels, and other ophthalmological anomalies. A weakness of this study is that from the original cohort, children who did not receive a FASD diagnosis were not subsequently followed up. This means that it is difficult to ascertain whether the incidence of ophthalmological anomalies is higher in FASD populations compared to matched controls.

In contrast to the study presented above, another relatively large-scale study (48) compared 43 PAE children with 55 control children between the ages of 4 and 9, and found that both

sampling groups exhibited an approximately equal incidence of arterial tortuosity (~15–16%). This is an important and contrary finding, because it points to a limitation in case-report-type studies; specifically, that there was limited-to-no assessment of the frequency of vessel tortuosity in 'control' populations. However, this negative finding does not by itself disprove the linkage between PAE and vessel tortuosity. Control populations may well represent the heterogeneity of outcomes following prenatal experience, and a number of other factors that were not controlled for, including, for example, hypoxia, plasma hyperviscosity and hypercoagulability, [for review, see (49)], may result in an identical outcome. Importantly, prematurity, a condition with multiple etiologies including PAE, is also associated with retinal vessel tortuosity (50).

It is probable that ocular blood vessel pathology may not be a unique feature of FASD, as documented in an earlier 1999 study of Swedish children with different developmental complications. In that study by Hellstrom (43), children with a diagnosis of FAS (n = 16) were compared to those with other developmental complications, including preterm birth (n = 39), periventricular leukomalacia that is typically associated with perinatal hypoxia/ischemia (PVL, n = 17), and with septo-optic dysplasia with optic nerve hypoplasia and pituitary hormone insufficiency (n = 6). Children in these groups were compared to a cohort of "healthy white Swedish children" (n = 100). Digital image and fundoscopic analyses were used to examine optic nerve and retinal vessel morphology. In all groups, the study documented significantly increased tortuosity of retinal vessels (above the median for the reference cohort) and a lower number of branching points (below the median for the reference cohort). While this study showed that ocular vessel pathology was associated with a number of developmental pathologies, ~43% of children with a diagnosis of FASD scored above the 95th percentile for the reference group. This outcome indicates that children with FASD are more likely to have retinal vessel pathology that the general population. This proportion was also larger than that for children with preterm birth. Contrary to the studies by Gyllencreutz et al. and Flanigan et al., discussed above, one of the strengths of this study was that FAS participants (median gestation age of 38 weeks at birth) and premature-birth participants (median gestational age of 29 weeks at birth) were separated for analysis. A weakness of this study was that the FAS sample in this study was poorly defined, except to refer to the then-current diagnostic guidelines as outlined by Sokol and Clarren (51). However, the Hellstrom study did support the specific linkage between a diagnosis of FAS and retinal vessel tortuosity, but also documented the linkage between other developmental anomalies and the same outcome.

In general, the findings from human studies support strong associations between prenatal alcohol exposure and vascular deficits, but cannot definitively advance a causal relationship between exposure and outcome. Studies of PAE in animal models, therefore, have a vital role in providing evidence for causality.

Animal models of PAE

Early studies in animal models convincingly show that PAE is a causal factor in the cardiovascular defects that were described in human populations. For instance, in a 1986 study, Daft et al. (52) reported that two doses of ethanol on gestational day 8 in a pregnant mouse resulted in cardiac septal defects and defects in the cardiac outflow vessels, including the aorta. Moreover, these defects persisted and could be observed 10 days later, suggesting that the effects of an episode of PAE were permanent. A second publication from 2002 reported that PAE throughout gestation in a rat model, albeit at much lower levels than those reported in the previous study (~24 mg/dL), resulted in a diminished vasoconstriction response of aortic rings following acute treatment with norepinephrine (53). The observed effects of PAE on the vasoconstrictive response were particularly strong when the arterial endothelium was intact, suggesting that the endothelium itself was a direct target of PAE. Interestingly, the study authors observed that PAE also resulted in diminished vasodilative response to the cholinergic mimetic carbamylcholine chloride, suggesting a broader impact of PAE on the adaptability of large arteries to physiological demand. Collectively, these data identify PAE, over a range of doses and exposure times, as a causal factor in the development of persistent structural and functional cardiovascular defects.

Other studies in primate (54), ovine (55), and rodent models (56) also point to placental and uterine blood flow as targets of prenatal ethanol, relating vascular deficiencies in these tissues to decreased fetal growth (see Table 2). However, cardiac defects due to developmental ethanol exposure have also been documented in non-placental vertebrate models, like zebrafish (66), suggesting that ethanol's effects on cardiovascular development are not exclusively mediated by potential utero-placental insufficiency. Again, these studies support a causal link between PAE, decreased peripheral blood flow, and subsequent deficits and brain growth. However, the first studies that specifically investigate PAE effects on brain vasculature were not published until approximately 30 years after FAS was first described in human populations (7) and ~25 years after it was first described in the United States (6). This delay in research modeling alcohol's effects on brain vasculature speaks to the neural cell-centric focus of the research field at that stage.

Early assessments of cerebral circulation in animal models of PAE

In 1997, Gleason et al. at Johns Hopkins University, School of Medicine used a sheep model of PAE (57), in which pregnant

TABLE 2 Studies on the vascular effects of PAE in animal models.

Species	Study type	Vascular region	Major findings	References
Sheep	*In vivo* and *in vitro*	Brain	PAE in early gestation resulted in decreased postnatal cerebral blood flow in labs, in response to both hypo- and hypercapnia, compared to controls	(57)
Rat	*In vivo* and *in vitro*	Aorta	PAE alters aortic vascular contractile function, decreased vasoconstrictive response to norepinephrine and potassium chloride	(53)
Sheep	*In vivo*	Brain	In the presence of acidemia and hypercapnia, without hypoxia, there was increased blood flow to brain in sheep that were exposed to moderate alcohol levels during late gestation	(58)
Sheep	*In vivo*	Brain	PAE in mid gestation significantly attenuated dilatory cerebral blood flow response to hypoxia	(59)
Sheep	*In vivo*	Brain	PAE resulted in increased maximum vasodilation of fetal cerebral arterioles and vessel response to selective A2A adenosine receptor agonist and to acidosis	(60)
Sheep	*In vivo*	Brain	PAE in mid-gestation increased the dilatory response of the adult intracerebral arteries due to VIP but had no difference in response to pH or myogenic tone	(61)
Sheep	*In vivo*	Brain	PAE in the mid-gestation resulted in lower brain weight, but no significant differences in cerebral microvessel density	(62)
Sheep	*In vitro*	Uterine	Isolated uterine endothelial cells from pregnant ewes, exposed to binge-like ethanol levels. Observed decreased eNOS expression, phosphorylation and expression of eNOS related proteins	(55)
Mice	*In vivo*	Placental, cardiac	Binge-like PAE in pregnant mice at gastrulation persistently increases vascular resistance in umbilical artery, cardiac valvular regurgitation and isovolemic relaxation time	(63)
Mice	*In vivo*	Brain	Single and repeated binge-like PAE in mid- to late-pregnancy, during the peak period of cortical neurogenesis results in persistent decrease in cardiac output through umbilical and cerebral arteries	(64)
Mice	*In vivo* and *ex vivo*	Brain	PAE in the late gestation resulted in disorganized cerebral microvascular networks, including reduced density of cortical vasculature, decreased VEGF and VEGF receptor mRNA and increased VEGF receptor (R1) expression and *ex vivo*, decreased in microvessel plasticity	(35)
Rat	*In vivo*	Uterine	An episode of PAE in both early and late pregnancy decreased acetylcholine-induced uterine artery vasodilation	(65)
Zebrafish	*In vivo*	Cardiac	Exposure during embryogenesis resulted in a dose-related increase in irreversible damage to dorsal aorta, segmental artery coarctation, and motor function deficits	(66)
Primate	*In vivo*	Placental	PAE in early pregnancy in Rhesus monkeys significantly decreased placental perfusion and oxygenation in fetal vasculature in later stages of pregnancy	(54)
Primate	*In vivo* and *ex vivo*	Brain	Exposure of fetal MCAs to alcohol in mid pregnancy induces increased dilation of cerebral arteries and peak systolic velocity, mediated by vascular endocannabinoid receptors	(67)
Mouse	*In vivo*	Carotid artery	The carotid arteries of adult mice with PAE exhibited significantly decreased blood acceleration with loss of blood flow to the brain in the long term, and was associated with decreased recovery from cerebrovascular stroke	(68)
Mouse and Human	*In vivo*	Placenta, Brain	Deficiency in placental angiogenic factor implicated in VEGF (vascular endothelial growth factor)-receptor mediated deficiencies in brain angiogenesis	(69)
Primate	*In vivo* and *ex vivo*	Eye	PAE resulted in increased intraocular pressure (IOP) in juvenile and adult offspring, increased fundal tessellation indicative of abnormal choroidal vascularization, and astrocytosis	(70)
Primate	*In vivo*	Brain	Peak systolic velocity and pulsatility index of anterior and middle cerebral arteries decreased during episodes of alcohol intoxication, with decreased fetal cerebral artery Doppler indices	(71)
Rats	*In vivo*	Uterine	Uterine arteries from alcohol exposed rats had reduced acetylcholine-dependent relaxation and impaired endothelial nitric oxide signaling	(72)
Mouse	*In vivo*	Brain	*In utero* speckle variance optical coherence angiography showed that binge-like PAE caused rapid and significant cerebral microvessel constriction compared to controls	(73)
Primate	*In vivo*	Brain	PAE in mid pregnancy resulted in significant increases in transferase and oxidoreductase class proteins and increased ALDH activity in fetal cerebral basilar arteries	(74)
Primate	*In vivo* and *ex vivo*	Brain	PAE induced fetal artery dilation mediated by cannabinoid signaling is transient and does not persist to the end of pregnancy	(75)
Rat	*In vivo*	Brain	PAE throughout pregnancy resulted in decreased nitric oxide dependent dilation of cerebral arterioles, higher superoxide levels, increased brain infarct volume following cerebrovascular ischemia, and increased levels of superoxide	(76)
Rat	*In vivo*	Brain	PAE throughout gestation resulted in decreased stimulus-dependent vasodilation response in cerebral arteries in young adult offspring	(76)

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TABLE 2 (*Continued*) Studies on the vascular effects of PAE in animal models.

Species	Study type	Vascular region	Major findings	References
Mouse	*In vivo*	Brain	*In utero* ethanol-exposure resulted in an acute-onset dose-dependent decrease in cerebral microvessel diameter and decreased blood flow measured by correlation mapping optical coherence angiography. Concurrently the maternal femoral artery exhibited vasodilation	(77)
Mouse	*In vivo*	Umbilical	PAE in the latter half of gestation resulted in intrauterine growth retardation, and diminished umbilical arterial blood flow, assessed by pulse-wave Doppler ultrasound. RNA-seq analysis of the placenta, this study found diminished expression of placental genes for hematopoiesis and chemosensory pathways	(56)

ewes received either an alcohol or saline infusion daily for 3 weeks during early gestation (~Gestational Day 31). This exposure resulted in mean blood ethanol concentrations of ~167 mg/dL after 1 hour. Physiological characteristics of cerebral blood were ascertained by sampling from the superior sagittal sinus of instrumented PAE and control newborn lambs, and blood flow was quantified using infusions of radionuclide-labeled microspheres. The authors reported that both the hypo- and hypercapnic cerebral blood flow response was decreased in PAE lambs compared to controls.

Subsequently, Parnell et al. at Texas A&M University (58) and Mayock et al. at the University of Washington (59) published the next preclinical studies on the effects of PAE on blood flow in the fetal brain, also using an ovine model. Notably, in both studies, assessments of PAE effects were conducted in the fetus, i.e., more proximate to the exposure period, rather than postnatally, as reported above by Gleason et al. In the study by Parnell et al., pregnant ewes were exposed to ethanol between gestational days (GD) 109–132, resulting in maternal blood ethanol concentrations between 85–185 mg/dL. The authors also used radionuclide-labeled microspheres to assess cerebral blood flow, and found increased retention of radionuclides in the fetal cerebellum, indicative of increased blood flow, but only at the highest levels of exposure.

Studies by Mayock et al. at the University of Washington, Seattle, also used an ovine model to assess the effects of PAE on brain vasculature (59, 60). In their studies, Mayock et al. exposed pregnant ewes to alcohol at an earlier time frame than the previously cited paper by Parnell et al. PAE occurred *via* daily intravenous infusion between GD 60–90, equivalent to the 2nd trimester period in human pregnancy. Post-infusion maternal alcohol levels peaked at ~200–214 mg/dL, which although high, are within the range of levels attained by individuals with alcohol use disorders [e.g., see (78)]. In their 2007 study, Mayock et al. utilized a similar radioisotope retention paradigm to assess cerebral blood flow as used by Parnell et al. However, this study found decreased cerebral blood flow due to PAE, opposite of what was previously reported.

In their subsequent 2008 report (60), Mayock et al. isolated penetrating fetal cerebral arterioles arising from the pial surface of fetal lambs between GD 125–128, investigating the persistent effects of on PAE offspring compared to control, saline infusion-exposed fetuses. The authors reported two key findings: more than 5 weeks after the final exposure, cerebral arterioles from PAE fetuses exhibited 1) a significant dilatory response to decreased pH, and 2) an increased maximal dilatory response to an Adenosine A2 agonist, CGS-21680. A follow-up study by the same team (61), also in isolated fetal sheep cerebral arteries, reported on a similar PAE-induced increase in vasodilatory response, this time to vasoactive intestinal peptide, suggesting that PAE may result in a general enhancement of stimulus-dependent cerebral arterial vasodilation.

A more recent study in a non-human primate model of PAE used an *ex vivo* model of pressurized fetal cerebral arteries (67), demonstrating that acute ethanol exposure also results in rapid vessel dilation. In this primate study, the authors were not able to document additional effect of prior intragastric PAE directly on the dilation response, though they did document increased sensitivity to cannabinoid signaling due to PAE. However, this last study also used a more limited exposure—just three episodes of intragastric gavage during the 2nd trimester-equivalent period of human pregnancy—and the peak maternal blood alcohol levels attained were ~80 mg/dL, substantially lower than levels attained in the first two studies.

Collectively, the aforementioned studies demonstrate that PAE can influence cerebral blood flow regardless of whether exposure occurred in early, mid- or late pregnancy. Moreover, vascular effects can persist beyond the period of ethanol exposure, into the neonatal period. Importantly, they also showed that vasodilation, via ethanol or other dilatory stimuli, was a long term response to PAE in cerebral blood vessels. However, the outcome of PAE for brain circulation itself is still unclear, since the radionuclide retention studies by Parnell et al. (58) were interpreted by the authors to suggest increased flow (at least in the posterior, cerebellar circulation), whereas Mayock et al. (59) and earlier, Gleason et al. (57), interpreted their data to indicate decreased blood flow, and under conditions of increased demand, reduced oxygen delivery within the brain. Some of the differences in study outcomes reported above, may well be due to inter-study

differences in the developmental timing of alcohol exposure and outcome assessment, as well as brain regional differences. It should also be noted that, radionuclide-labeled microsphere retention methodology which was historically, the gold standard for assessing end-organ vascular perfusion, was nevertheless subject to interpretive limitations. Based on the empirical association between afferent arterial tone and end-organ perfusion, increased brain retention of radio-label is causally linked to peripheral arterial vasodilation (57, 58). However, this interpretation requires some caution, since it does not account for potential dilation of efferent vessels, microcirculatory arterial-venous anastomotic shunts which are present in brain parenchyma [e.g., see (79)], that may result in increased clearance and decreased tissue retention of radiolabel (80), accounting for some discrepancies in study outcomes outlined above. Recent studies have used more direct, *in vivo* imaging modalities such as ultrasound imaging and optical coherence tomography to assess brain blood flow in response to PAE.

Ultrasound studies of major cerebral arteries in animal models

Studies using ultrasound imaging in primate (67, 71) and rodent models (64, 68) have been successful in visualizing flow parameters in large cerebral arteries like the anterior (ACA), middle (MCA) and posterior (PCA) cerebral arteries. Both primate and rodent models indicate that the immediate and persistent effect of PAE is decreased cerebral blood flow as measured by peak systolic velocity, or velocity time integral [VTI, a composite index that is a measure of the cardiac output through the assessed cerebral vessel (81)]. However, studies in primate models generally indicate that the effects of PAE do not persist past the exposure period, and specifically, not to pregnancy term. For example, Tobiasz et al. (71) found that fetal baboons exposed to PAE experienced decreased systolic velocity in anterior and middle cerebral arteries during the acute period of intoxication, but that these vascular effects did not persist through gestation. However, in rodent (mouse) models, Bake et al. showed that the PAE effect of decreased velocity, and decreased VTI could persist through gestation (64). In contrast to the acute fetal effects of PAE, Bake et al., in a follow-up study (68), showed that young adult PAE offspring (3 months of age) exhibited significantly increased carotid artery VTI, indicative of a vasoconstrictive response. It is certainly possible that species differences may have contributed to differences in the persistence of the vascular effects of PAE, though the observation of a peripheral hypertensive phenotype in human populations of children and teenagers with FASD (46), is consistent with increased carotid VTI in young adult PAE offspring in the above mouse study, and argues against a role for species differences. A second and plausible explanation is that the persistence of PAE effects had more to do with the dose and frequency of ethanol exposure. Blood alcohol levels of ~80 mg/dL were attained in the primate studies, for example, with exposures spaced 10 days apart (71), whereas BACs of 117–150 mg/dL were reached in the mouse, with up to two exposures per day for a 4-day exposure window (68). It is possible therefore that the cumulative exposure in the mouse model was significantly higher than that attained in the primate model. Nevertheless, both models show that, at least immediately, there is a net decline in fetal cerebral circulation due to PAE, consistent with the hypothesis that, in the short term, alcohol induces vasodilation (71). It is important to note that Bake et al. also followed PAE and control offspring into mature adulthood (12 months of age), where PAE had a diametrically opposite effect compared to that observed in young adults, i.e., resulting in decreased VTI compared to control offspring (68). This outcome suggests that in the long term, PAE may result in cranial vascular hypoperfusion in middle-aged adults, though at this time, there is no comparable data on vascular health in middle-aged persons with FASD.

Analysis of cerebral microcirculation in animal models

More recently, optical coherence angiography has been used to document the effects of PAE on blood flow through even smaller cerebral arteries and arterioles, including those that serve as tributaries from the ACA and MCA (73, 77). These studies report that PAE results in a rapid and dose-dependent decrease in the diameter of fetal cerebral micro-vessels, whereas the maternal femoral artery experienced vasodilation in the same time frame, as expected. It is likely that the vasoconstrictive response of cerebral microvessels is a compensatory adaptation to the dilation of larger and afferent vessels, as a means to maintain pressure in the microvascular network. This compensatory effort may be at least partly successful, as suggested by the increased radionuclide retention in the cerebellum that observed by Parnell et al. previously [as outlined above (58)].

Anatomical studies in mouse models also provide supporting evidence that PAE results in persistent damage to the intracerebral microvasculature. For instance, Jegou et al. (35) used a PAE model, albeit with heavy ethanol exposure, daily through the second half of murine gestation, and observed that mice with PAE had reduced density of cortical vasculature and disordered micro-vessel orientation, which were normally radially-oriented in control mice progeny. These data cumulatively suggest that even if the intracerebral microvasculature adapt and compensate for PAE, the immediate and long-term outcomes are likely to be vascular insufficiency within the brain. It will be important to ascertain whether this insufficiency extends to the availability of collateral circulation between terminal branches of cerebral arteries like the MCA, ACA and PCA. The evidence suggests that collateral circulation may also be impaired, and that vascular

insufficiency may also result in long-term adverse consequences to adult-onset brain diseases. For instance, two studies have documented the PAE results in decreased neurological recovery from an episode of cerebrovascular ischemic stroke (68, 76). While the study by Bake et al. (68) did not document companion damage to brain tissue despite neurological deficits, the study by Canzi and Mayhan (76) showed that ischemia did result in increased stroke volume in PAE offspring. Therefore, it is likely that compensatory collateral circulation is also impaired following PAE. Importantly, collateral circulation can emerge rapidly, during the period of cerebral arterial occlusion itself (82), suggesting the presence of latent collateral tributaries within leptomeningeal tissues. It is likely, though there is a need for further investigation, that latent collateral circulation is also impaired in PAE, leading to worse stroke outcomes.

Retinal imaging in animal models

As with human studies, recent studies in animal (primate) models, have also focused an imaging retinal vasculature to assess the effects of PAE on brain blood flow in adult offspring. One study in a large cohort of vervet monkeys (29 PAE and 20 controls), maintained as a community (70), investigated the effect of exposure of ethanol *in utero* on retinal abnormalities and premature aging of the retina using *in vivo* examination of the fundus and intraocular pressure (IOP), as well as a number of physiologic and anatomic measures. This study had well-defined and extensive data on maternal alcohol consumption for each pregnancy, with a range of exposure from 1.2 to 5.51 g of alcohol/kg body weight/day from mid-pregnancy to term. The authors found that PAE resulted in increased IOP, increased fundal vascularization, and the retina showed increased evidence for astrocytosis, particularly in the retinal ganglion cell layer and optic nerve, suggesting retinal damage. This is potentially an important finding, since increased IOP may be indicative of ocular hypertension, a condition linked to decreased ocular blood flow and thinning of the choroid plexus in human populations (83), and to increased risk for glaucoma (84). Collectively, the studies cited earlier in human populations, as well as the above study by Bouskila et al., make a case for monitoring ocular health in FASD populations, not only to decrease the risk for diseases like glaucoma, but potentially as a biomarker for neurovascular health.

Mechanisms that mediate effects of PAE on the vascular system

Several studies, including many cited above, have taken the first important steps to uncover mechanisms of ethanol toxicity. One consensus finding is that PAE alters the vasodilation response to G-protein receptor coupled signaling mechanisms and nitric oxide signaling [e.g., (53, 59, 60, 67, 72, 85)], as well as other environmental stressors (76). Furthermore, studies do support the theory that PAE also interferes with the trajectory and development of brain microvessels. This implicates the potential role of angiogenic factors, such as signaling through the vascular endothelial growth factor (VEGF) system. Lecuyer et al. (69) found evidence for decreased VEGF signaling and decreased brain angiogenesis due to diminished levels of placental-derived VEGF family member, PLGF (Placenta Growth Factor). Interestingly, PLGF supplementation was able to ameliorate the effects of PAE in their mouse model. Autophagy has also been implicated as a molecular mechanism in the potential remodeling of cerebro-vasculature following PAE (36) in both human studies and mouse models. Autophagy is an important stress response to vascular injury (reviewed in (86), and a potential target for therapeutic intervention. However, much more research is needed to identify and track mechanistic linkages between PAE and brain vascular outcomes.

Conclusion and limitations

Although the research on PAE effects on developing vasculature, and in particular brain vasculature, are somewhat sparse, both human studies and studies in animal models document the deleterious and persistent effects of PAE on cerebrovascular structure and function. The current research studies are based on relatively small sample sizes, but collectively provide a preponderance of evidence pointing towards several important pathways related to vasodilation, oxidative stress, and flow dynamics. It is also clear that vascular deficits are likely to persist through the lifetime and contribute to risks for adverse outcomes following adult-onset disease, like cerebrovascular stroke. In this context, one study which documented significantly increased risk for hypertension in children and adolescents with a diagnosis of FAS or pFAS (46) is particularly important, since it suggests that the risk for adult-like cardiovascular disease may appear earlier in persons with FASD compared to the general population, and further implicates pituitary and renal dysfunction in the pathogenesis of FASD. For instance, studies in human populations have linked prenatal alcohol exposure to hypermethylation of the proopiomelanocortin (POMC) locus (87), and in animal studies, PAE has been shown to result in deficits in feed-back inhibition and hyperresponsivity of the hypothalamic-pituitary-adrenal (HPA) axis in affected offspring [e.g., see (88)]. Animal studies have also shown that PAE results in loss of renal nephrons and increased blood pressure in exposed offspring (89). Collectively these data suggest that hyperresponsiveness of the HPA axis and renal deficiency may contribute to vascular pathology in FASD, and support the need for further studies on the intersection between endocrine, renal and vascular function.

Human studies document disruption to mammalian target of rapamycin (mTOR) pathways, with increased autophagic

vacuoles in brain microvessels, deficits in mitochondrial pathways, and basement membrane adaptations, including capillary basal hyperplasia and endarteritis. All of these outcomes are compatible with a stress-related remodeling of tissue microvasculature following PAE that likely results in compromised vascular function. Animal models further provided evidence that PAE is a causal factor in vascular deficits, including those of the cerebral vasculature. Additionally, preclinical research has identified PAE-affected mechanisms of signaling pathways that target vasodilation, such as the endocannabinoid receptor system, prostacyclin and nitric oxide pathways, and calcium mobilization. Other factors, such as impaired VEGF receptor signaling and changes in protein expression related to mitochondria and oxidative stress, may contribute further to structural pathologies.

Our analysis of the literature also identified studies in both human and animal populations that assessed ocular vascular function following PAE. Studies in FASD populations documented other ophthalmological findings, such as increased retinal vessel tortuosity, which should be readily assessable in primary healthcare settings. These studies make a case that routine, easy to accomplish, clinical assessments of ocular blood flow and vascular structure may be useful as a proxy marker for neurovascular competency in persons with diagnoses along the FASD continuum. Additional studies are needed to determine whether such routine ocular assessments have predictive value for the management of adult-onset cerebrovascular disease in FASD populations. However, attention to cardiovascular and cerebrovascular health in FASD populations is likely to be helpful in managing both early cognitive and neurobehavioral deficits as well the delayed, adult consequences of PAE.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prenatal exposure to alcohol: mechanisms of cerebral vascular damage and lifelong consequences

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Alcohol is a well-known teratogen, and prenatal alcohol exposure (PAE) leads to a greater incidence of many cardiovascular-related pathologies. Alcohol negatively impacts vasculogenesis and angiogenesis in the developing fetal brain, resulting in fetal alcohol spectrum disorders (FASD). Ample preclinical evidence indicates that the normal reactivity of cerebral resistance arterioles, which regulate blood flow distribution in response to metabolic demand (neurovascular coupling), is impaired by PAE. This impairment of dilation of cerebral arteries may carry implications for the susceptibility of the brain to cerebral ischemic damage well into adulthood. The focus of this review is to consolidate findings from studies examining the influence of PAE on vascular development, give insights into relevant pathological mechanisms at the vascular level, evaluate the risks of ethanol-driven alterations of cerebrovascular reactivity, and revisit different preventive interventions that may have promise in reversing vascular changes in preclinical FASD models.

KEYWORDS

brain, cerebral resistance arterioles, prenatal alcohol exposure, FASD, cerebral blood flow

Introduction

One of the most often used psychotropic substances, ethanol, permeates the placental barrier, and hence exerts potential teratogenic effects. A 2011 survey found that 45 percent of pregnancies in the United States were unintentional, with one in ten pregnant women between the ages of 18 and 44 reporting intake of alcohol in the previous 30 days and one in thirty-three reporting binge drinking [1, 2]. The pattern and intensity of the detrimental effects of prenatal alcohol exposure are dependent on alcohol dose, timing, sequence, and persistence of alcohol intake. All current research indicates that alcohol has a detrimental effect on fetal development. However, the fetal brain is the most substantially afflicted organ, displaying structural and functional abnormalities as a result of maternal alcohol consumption [3, 4]. Exposure to alcohol may result in fetal alcohol spectrum disorders (FASDs) in humans. FASD is a collective name for the harmful effects of prenatal alcohol exposure (PAE), including impairments in physiologic, neurologic, and behavioral development [5]. It should also be noted that FASD is an umbrella term

that incorporates a variety of alcohol-related disorders with a range of severity. The most severe type of FASD, fetal alcohol syndrome (FAS) is characterized by specific physical traits in neonates. Partial FAS (pFAS), on the other hand, is diagnosed when there is validated prenatal alcohol exposure but not enough signs and symptoms to confirm a diagnosis of FAS [6]. Recent investigations, however, have seen the effects of alcohol on fetal cerebrovascular function emerge as a key mediator since brain energy needs are usually fulfilled by a continually adjusting blood supply [7]. Such an adaptation begins at the level of cerebral arteries and extends to microvessels that enter the brain parenchyma and form the neurovascular unit [8,9]. PAE may have acute and chronic teratogenic and toxic impacts on cerebrovascular physiology. This review's objective is to outline the pathological pathways in different developmental phases, as well as their morphological and functional consequences on the cerebral circulation. It is held that alterations of cerebral blood flow (CBF) owing to dysregulation of cerebral blood vessels in PAE may be a significant contributor to the etiology of several cerebrovascular events, such as stroke.

Cerebrovascular effects of PAE during fetal development

During the early-gestation period, the neural tube is surrounded by a perineural vascular plexus (PNPV) developed by vasculogenesis, the process of blood vessel development in the embryo, which involves the *de novo* generation of endothelial cells (ECs). Beginning in the mid-gestation period, endothelial cells from the PNPV penetrate the neural parenchyma, utilizing fibers from multipotent stem cells, like radial glia cells from the developing forebrain, to direct their migration toward the ventricular surface, thereby initiating the first vessels of the CNS. The second trimester is crucial for neuronal and blood vessel growth in the fetal brain [10, 11]. During this phase, a network of arteries inside the sub-arachnoid space gives birth to microvessels that enter the fetal brain [11]. This emerging vasculature supports nutritional requirements and endocrine regulation of fetal brain development [12]. In the early-mid gestation period, vascular development in the CNS is mediated by angiogenesis, which is defined as a series of cellular and molecular processes including the production of new vessels and culminates in the formation of the blood-brain barrier (BBB) [13,14,15]. At the mid-late gestation period, interactions among neural cells and ECs are initiated and continue through late-gestation and until birth. Communication between glial cells and the vasculature is crucial for the optimal development of the nervous system [16]. Pericytes increase vascular stability by releasing a variety of stabilization factors, such as angiopoietin-1 (ANG1) and platelet-derived growth factor (PDGF-β), tissue inhibitor of metalloproteinase 3 (TIMP3) [17]. In response to neural impulses at the perivascular terminal, astrocytes produce chemicals capable of regulating vascular tone. From the late-gestation period until birth, the BBB continues to mature *via* the expression of various molecules by the BBB's cellular components, culminating in the development of a basal membrane rich in laminin, collagen IV, and fibronectin that fully surrounds the brain vasculature [18].

It has been established that maternal ethanol exposure causes rapid and persistent loss of blood flow from the umbilical artery to the fetal brain, potentially distressing nutrition and the maternal/fetal endocrine environment during a critical stage for neurogenesis and angiogenesis in the developing brain [19]. Because alcohol exposure during pregnancy is known to alter brain development, the majority of investigations have centered on neural cells. At the molecular and subcellular levels, however, cerebrovascular development is a more complicated, multi-step process involving a large number of molecular participants.

Angiogenesis is a complex process that is delicately controlled by a balance between pro-and anti-angiogenic factors [20]. They consist of, but are not limited to, vascular endothelial growth factor (VEGF), integrins, fibronectin, angiopoietins, vascular cell adhesion molecules (VCAM), fibroblast growth factors (FGF), tyrosine kinases (TK), extracellular matrix (ECM) proteins and proteinases, transforming growth factor (TGF), and Wnt signaling factors as growth-stimulatory signals. On the other hand, tumor necrosis factor (TNF) signaling and pro-apoptosis factors are stop signals [21, 22, 23]. Recent research conducted on adult nonhuman primates [24] and mice [25, 26] conclusively demonstrates that alcohol has a deleterious effect on vasculogenesis and angiogenesis pathways. According to microarray investigations, genes (e.g., VEGF gene family) implicated in angiogenesis are molecular targets of ethanol toxicity [27].

In addition to disrupting the signaling components that regulate vascularization and brain development, PAE may affect molecular targets that are particular to cerebrovascular development. Ethanol may affect the molecular and cellular elements of the BBB directly as soon as they are present during CNS development. Other research indicated that the embryonic brain is more susceptible to ethanol due to the high prevalence of proapoptotic proteins and low expression of proteins associated with stress response systems, namely autophagy or unfolded protein response [28]. Also, a proteomics investigation revealed that alcohol-exposed baboon fetuses mainly had changes in mitochondrial and structural proteins in their cerebral arteries. Unlike mitochondrial proteins, structural proteins were downregulated in the brain of fetal arteries exposed to alcohol [29].

Morphological damage in cerebral blood vessels due to PAE

Considering ethical limits of human-based research, the PAE of laboratory animals has been extensively used as an alternative. Results from these animal models showed a significant influence on the generation and expression of microvessels in the rodent model. Besides dose, the timing of ethanol exposure has a significant impact on the outcome of fetal brain development. Since the gestational period of rodents (i.e., 18–23 days) is much shorter than that of humans, the morphological and functional effects of alcohol on these animals may need to be extrapolated to equivalent pregnancy terms in humans. In both rats and mice, gestational day (GD) 1–10, GD 10–20, and postnatal days (P) 1–10 can be considered as the equivalents of first, second, and third trimester human pregnancy, respectively [30]. Prior research on rat models exposed to moderate prenatal alcohol dosages revealed ultrastructural changes in brain capillaries at P20–30 [31]. In a study using rat models, the oral administration of 6.6 g/kg of ethanol from P4 to P10 was evaluated at P10 for any alterations in brain microvasculature [32]. There was no change in capillary density, while capillary diameters were increased in the cerebellum and hippocampus regions, unlike the dentate gyrus region [32]. Later, in a mouse model (at P2) of the third trimester equivalent of human pregnancy demonstrated the loss of radial orientation of the microvessels and a reduction in cerebral vascular density when maternal injection of 3 g/kg ethanol occurred during GD 13–19, which was a time-line equal to the second trimester of human pregnancy [33]. Overall, these rodent studies suggest that alcohol exposure during the human mid and late trimesters might affect the microvasculature differentially in the brain regions.

Human embryos are susceptible to ethanol. Evidence suggests that exposure to high amounts of ethanol during human development causes craniofacial, cardiovascular, and neurological abnormalities, which are often accompanied by cognitive and behavioral deficiencies. Autopsy and magnetic resonance imaging (MRI) investigations found that individuals who were exposed to alcohol *in utero* had structural brain damage, including lower brain sizes and reduced amounts of white and gray matter inside the brain [reviewed in 4]. However, the animal research mentioned earlier suggests that exposure to PAE during late gestation might be perilous. In a postmortem examination of human brain tissues from fetuses that deceased spontaneously *in utero*, stage-dependent alterations in the cortical vascular network were detected in the cortex of fetuses with FAS. The radial arrangement of cortical microvessels was markedly disrupted in FAS patients after 30 weeks of gestation, whereas no changes were seen in alcohol-exposed human embryonic brain tissues between 20 and 22 weeks of gestation. In addition, dynamic microscopy techniques indicated that alcohol altered endothelial cell activity and survival as well as the plasticity of the microvessel [33].

In conclusion, investigations conducted on rodents and humans suggest that prenatal alcohol consumption, especially during the late stages of gestation, may affect the macroscopical or microscopical structure of the cerebral microvascular network. These age-dependent abnormalities can be theorized because of a disturbance in cortical angiogenesis.

Functional changes in cerebrovascular circulation due to PAE and their possible implications

Critical to brain function is a coherence among the metabolic needs, the supply of oxygen and nutrients, and the elimination of cellular waste. This matching requires continual modulation of CBF, which may be divided into four basic categories: autoregulation, vascular reactivity, neurovascular coupling (NVC), and endothelium-dependent responses. Due to the limited scope of this paper, this part of the review will discuss how PAE affects the functional parts of cerebrovascular circulation, with focuses on how it changes CBF and vascular reactivity and the clinical effects of these changes.

Changes in CBF

The effects of maternal alcohol intake during pregnancy on outcome depends on the quantity and patterns of alcohol consumption. In animal trials, binge-like drinking patterns, in which the unborn is exposed to elevated blood alcohol concentrations (BACs) during relatively brief intervals, were shown to be more detrimental, even when the overall quantity of alcohol taken was lower than that of more continual drinking patterns [34, 35, 36]. Because binge drinking produces high BACs, may occur at important phases of brain development, and can be coupled with recurrent withdrawal episodes, it may be extremely detrimental [35]. In addition to a single binge pattern, recurrent binge-like events of maternal ethanol consumption are harmful to the fetus likewise [35] and may cause transitory and chronic abnormalities in cerebrovascular functioning. Abrupt CBF alterations in embryos may be associated with craniofacial deformity, fetal growth limitation, neuronal death, impaired delivery of nutrients to and elimination of metabolites from neurons, and a reduction in cerebral arterial tone. Here, we will examine how varied patterns of maternal alcohol use affect the blood flow to the cerebral arteries.

Temporary effects of maternal alcohol consumption on CBF

Following acute ethanol consumption, previous research on pregnant murine [19], ovine [41, 40, 39, 38, 37], and baboon [46,

45, 44, 43, 42] have showed aberrant uterine and cerebral blood flow. During acute maternal alcohol intake *in vivo*, the majority of investigations observed an increase in fetal cerebral perfusion and, perhaps, a decrease in fetal cerebral artery blood flow doppler velocity indices [46, 38, 42, 19]. In contrast, maternal infusions of 1 g/kg ethanol were associated with a reduction in CBF in preterm sheep [39] and baboons [45]. There was no decline in fetal CBF in mid-gestation sheep [40]. In this trial, 1 g/kg of ethanol was infused into the maternal blood at gestational day 92 (human equivalent term at 145–150 days), resulting in a maternal BAC of 150 mg/dl [40]. In contrast, in a separate experiment using the same animal model and a comparable experimental technique, Parnell et al. (2007) found that a greater dosage of ethanol (1.75 g/kg) substantially enhanced CBF by over 30 percent. Specifically, in the cerebellum, the rise in CBF 1 h after ethanol infusion was up to 50 percent greater than in the control group. These two experiments together demonstrated that, *in vivo*, the effects of alcohol on cerebral blood flow were concentration-dependent and brain region-specific.

In addition, the alterations in fetal CBF were detected along with the changes in systemic hemodynamics: a considerable increase in fetal cardiac output and heart rate, as well as a decrease in mean arterial pressure and systemic peripheral resistance [41]. An ultrasonography investigation demonstrates that acute single and recurrent binge-like episodes of maternal ethanol intake may promptly and chronically alter cranially-directed fetal blood flow throughout the second trimester [19]. PAE not only induces alterations in CBF but also impairs autoregulation. Additionally, PAE also modulates CBF responses to environmental variables. It was shown that fetal cerebral vasodilator responses to hypoxia [47] and acidosis [48] are altered by exposure to ethanol during the second trimester. Earlier work in an ovine model of pregnancy showed that PAE (1 g/kg ethanol maternal infusion i.v. for 3 weeks during the equivalent of the first trimester) reduced the adaptive rise in CBF in response to hypoxia in 1–4-day-old term lambs. As a consequence, neonatal brain oxygen supply could not be sustained [49]. Thus, it demonstrates that PAE as early as the first trimester might increase cerebral vascular susceptibility to environmental damage. Changes in the blood's metabolic profile, such as acidemia and hypercapnia, often follow acute alcohol-induced disruption of CBF and may contribute to its clinical effects [41, 40, 39].

Persistent effect of maternal alcohol consumption on CBF: Impairment of vascular reactivity

Multiple groups have explored the influence of PAE on fetal cerebrovascular function. A study, utilizing doppler ultrasonography revealed that in a baboon model of pregnancy, diminution of PAE alcohol impact occurred earlier to delivery. That study particularly indicated that acute fetal alcohol intake, with a maternal BAC of 80 mg/dl in the equivalent of the second trimester of human pregnancy, decreased cerebral blood flow in fetal cerebral arteries. However, this influence on fetal vascular function did not persist throughout the duration of pregnancy [45]. This indicates that alcohol-induced changes in the physiological characteristics of the fetal cerebral arteries disappear with development. In another example of an ovine PAE model, the alcohol impact of PAE diminished after delivery. Adult cerebral artery dilatory effects to adenosine A2A receptor agonist, CGS21680, were investigated *in vitro* using arterioles extracted from third trimester comparable ovine fetuses exposed to ethanol *in utero*. The dilatory reaction to micromolar doses of CGS21680 was substantially greater when compared to control group [48]. However, when arterioles were collected from adult sheep using a similar ethanol administration approach [50], same dilator responses were comparable to the control counterpart. Therefore, experiments using diverse animal models suggested that PAE-induced abnormalities in the pharmacological nature of cerebral arteries were slowly reversed with aging.

Turcotte et al. found that prenatal exposure to ethanol (6.4%) lowered the relaxation of the aorta to carbamylcholine in rats 25 weeks after birth, indicating a change in the reactivity of peripheral arteries [51]. Similarly, in peripheral arteries, some of the alcohol-induced modifications in fetal cerebral artery characteristics may persist and be detectable long after birth. Multiple studies on rats reveal the long-lasting effects of PAE. *In vivo* responses of cerebral arterioles to eNOS- and nNOS-dependent agonists were reduced in young rats (4–6 weeks old) [52] and in adult rats (12–15 weeks old) [53,54] exposed to alcohol during fetal development. While it is known that prenatal alcohol exposure impairs the dilatation of cerebral arterioles in rats, no research has explored the effect of prenatal alcohol on the constrictor response of cerebral arterioles until recently. The response of cerebral arterioles to U-46619 (a thromboxane-mimetic analog) and arginine vasopressin (AVP), which are physiologically important constrictors, was comparable in male and female rats independent of prenatal alcohol exposure and age. Similarly, there was no difference between male and female adolescent rats' responses to angiotensin II after prenatal alcohol consumption. At adulthood, however, alcohol-exposed females demonstrated an unanticipated dilatation in response to a high concentration of angiotensin II, but males did not. Except in adult female rats, the majority of the vasoconstriction responses to prolonged prenatal alcohol exposure were retained [55]. Besides inducing the loss of vascular reactivity, PAE is capable of inducing arterial stiffness to the cerebral vasculatures [56].

Implications

Long-term research on humans have previously proven that offspring of binge-drinking mothers demonstrate

TABLE 1 Multiple cellular and molecular mechanisms of cerebrovascular impairment due to prenatal alcohol exposure.

Summary Table: Molecular and cellular mechanisms of cerebrovascular impairment due to prenatal alcohol exposure

❖ **Mechanism of transient effects of prenatal alcohol exposure:**

A. Cerebrovascular layer involved: Cerebrovascular myocyte (mainly)

- Through eCB system: CB receptor types 1 and 2 [46].
  - Preclinical agent used for improvement: Rimonabant, a CB receptor inverse agonist [46]

❖ **Mechanism of persistent effects of prenatal alcohol exposure:**

A. Cerebrovascular layer involved: Cerebrovascular myocyte

1. Through increasing anandamide (AEA)-induced dilatation [46].

B. Cerebrovascular layer involved: Cerebrovascular endothelial cells

2. Through increased expression of collagen and tropoelastin, leading to increased stiffness of the vessel [56]
3. Through eNOS and nNOS-dependent impairment, leading to decreased reactivity of the vessel [52, 53]
4. Through increased generation of ROS [52, 53]

–Preclinical agent used for improvement:

- Apocynin (non-specific action) [52, 53].
- Rosiglitazone (specific action) [54].

5. Through decreased 5-hydroxytriptamine mediated vasodilation [56].

C. Extravascular

6. Through neurohormonal alteration
   - E.g. reduces VIP levels [92, 50].

❖ **Mechanism of morphological modifications of NVC:**

a. Angiogenesis:

1. Through VEGF-mediated modification:

   Preclinical agent used for improvement: Exogenous VEGF [33].

2. Through inhibition of autophagy in brain endothelial cells:
   1. Enhanced expression of microtubule-associated protein (LC3) and ubiquitin-binding protein (p62) in endothelial cells [94].
   2. Downregulation of the mTOR pathway.
      - Preclinical agent for improvement: mTOR inhibitor, rapamycin [94].

b. Endothelial cell survival:

1. Through generation of ROS > mitophagy > cell death [96].
2. Through releasing of AIF > impaired mitochondrial integrity > cell death.
   - Preclinical agent used for improvement: Activation of autophagy by rapamycin [94].
3. Through Mitochondria-linked cellular apoptosis [97]
   - Preclinical agent used for improvement: ROS scavengers and antioxidants.

❖ **Mechanism of loss of integrity of NVC:**

1. Through enhanced permeability of BBB: anticipated (no published evidence yet).
2. Through MMP-induced proteolysis of the neurovascular matrix [99,100,101].

Expansion of abbreviations used: AEA, N-arachidonoylethanolamine; AIF, apoptosis-inducing factor; BBB, blood brain barrier; CB, cannabinoid; eCB, endocannabinoid; eNOS, endothelial nitric oxide synthase; LC3, 1A/1B-light chain 3; MMP, matrix metalloproteinases; mTOR, mammalian target of rapamycin; NVC, neurovascular coupling; nNOS, neuronal nitric oxide synthase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.

particularly significant cognitive and behavioral abnormalities. The ability of the brain to maintain adequate cerebral blood flow in the face of changes in metabolic demand may influence the pathogenesis of symptoms associated with FASD in adults, i.e., cognitive decline, psychiatric symptoms, dementia, and seizures, all of which may be directly impacted by the ability of the brain to maintain adequate cerebral blood flow (neurovascular coupling) [57–60].

In addition, the loss of cerebrovascular autoregulation, such as during severe hypertensive encephalopathy, may result in catastrophic occurrences, such as subarachnoid hemorrhage and hemorrhagic stroke [61]. Evidently, our lab has also

shown that in prenatal exposure to alcohol exacerbated brain damage in adult rats after ischemia/reperfusion and that treatment of dams with apocynin reduced this increase in brain injury following ischemia/reperfusion [53]. Thus, it may be inferred that PAE-induced alterations in cerebral blood flow not only contribute to the pathophysiology of fetal alcohol syndrome, but also have the potential to cause serious brain injury.

Molecular and cellular mechanisms of impairment and recent preclinical interventions

Because alcohol is a simple ligand that may concurrently target several chemical entities, its effects on the developing brain are very complicated. Alcohol may cause cell death in some types of brain cells while interfering with the cellular and molecular activities of other types. These effects may be caused by alcohol both directly and indirectly.

Alcohol may have direct effects on embryonic brain development by interfering with neuronal proliferation and migration [62] or by inducing cell death [63,64,65]. In addition, alcohol may raise fetal glutamate levels [66, 67] and decrease glutamate N-methyl-D-aspartate receptors [68, 69], which may result in aberrant neuronal and glial migration.

Alcohol-induced hypoxia in the fetus is a significant indirect cause of alcohol. Alcohol reduces blood flow to the umbilical artery [70, 71], which might result in growth retardation [72, 73]. In addition to inhibiting protein synthesis and altering hormone levels, alcohol may further impede development [74, 75]. Increased oxidative stress on the embryo [76, 77] and disruption of growth factor signaling [78, 79] are additional pathways.

These effects can be toxic (short-term) and teratogenic (long-term). This section will examine as well as summarize (Table 1) the toxic and teratogenic processes that generate short-term or long-term effects on the cerebral arteries and microvessel network.

Mechanism of transient and persistent effects of prenatal alcohol exposure

Alcohol's short-term effects on CBF are highlighted by ethanol's targeting of molecular players inside the fetal cerebral blood vessel. In particular, PAE is characterized by fetal cerebral artery dilatation in the presence of alcohol, which is mediated by cannabinoid (CB) receptors 1 and 2. A study showed that the endocannabinoid (eCB) system is a target of maternal alcohol intake inside the fetal cerebral arteries, and that rimonabant, a CB receptor inverse agonist, might be a potential rescue treatment, as suggested by Seleverstov et al. [46]. Furthermore, fetal CB2 receptor-mediated cerebral artery dilation by anandamide was up-regulated in alcohol-exposed fetuses, showing that these receptors are the primary determinants of the persistent vasodilatory impact of alcohol on fetal cerebral arteries [46]. In addition to the increase of anandamide (AEA)-induced dilatation, other mechanisms of PAE's lasting effects are described in subsections below.

Endothelial nitric oxide synthase

Endothelial nitric oxide synthase (eNOS) is extremely susceptible to prenatal ethanol exposure in fetal cerebral arteries. In fetal cerebral arteries of an ovine model of pregnancy - where fetal plasma alcohol concentration reached 108 mg/dl during the late gestational period of day 95–133 (human equivalent term pregnancy) - a decreased endothelium-dependent vasodilation was observed in response to the dilator 5-hydroxytriptamine in alcohol-exposed donors. Authors also discovered a substantial reduction in endothelial nitric oxide synthase (eNOS) mRNA [56]. In contrast, in adolescent rats (4–6 weeks old) and adult rats (12–15 weeks old), *in utero* alcohol exposure decreased cerebral arteriole responses to eNOS (ADP) and neuronal nitric oxide synthase (nNOS)-dependent (NMDA) agonists [52, 53]. However, in microvessels and tissue from the parietal cortex of adolescent rats, the expression of eNOS and nNOS levels was not altered [52].

Stiffness of the arterioles

In the ovine model mentioned above, Parkington et al. also discovered that PAE significantly increased the fetal cerebral artery's total functional stiffness. Evidently, the elastic modulus of arteries in alcohol-exposed groups was approximately double that of control groups. In comparison to the control group, the alcohol-exposed group showed considerably higher mRNA levels for collagen Ia1 and tropoelastin, revealing the mechanism behind the enhanced functional stiffness of the vessel [56].

Increasing production of Reactive Oxygen Species (ROS)

EtOH and its catabolite acetaldehyde are itself harmful, although oxidative stress is the predominant damaging mechanism, according to current understanding. Oxidative stress is characterized by high intracellular levels of reactive oxygen species (ROS) that cause lipid, protein, and DNA damage. ROS are molecules or ions generated by the incomplete reduction of oxygen by one electron. The principal ROS species are superoxide (O2•), hydrogen peroxide (H2O2), and the hydroxyl radical (•OH). Cananzi and Mayhan evaluated the production of superoxide in the cerebral arterioles of prenatally alcohol-exposed adolescent and adult rats [52, 53], given that oxidative stress has been found to enhance neurovascular and neuronal damage and death in multiple brain locations in FASD (Figure 1A). PAE increased the

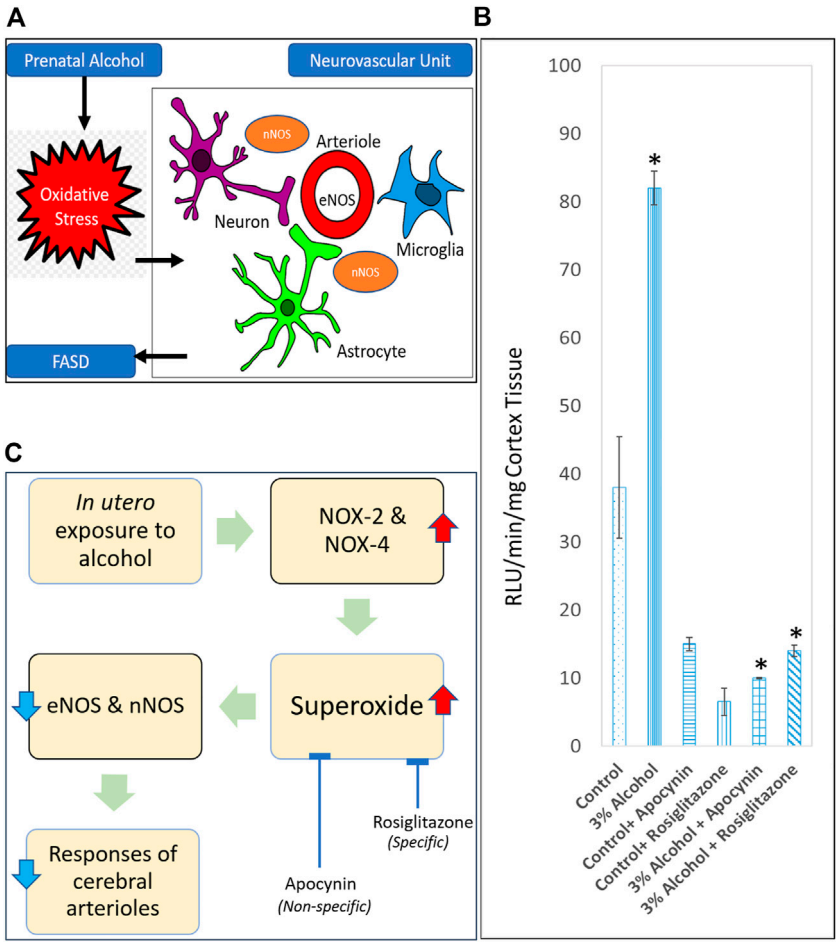

FIGURE 1
Role of oxidative stress in causing neurovascular damage in FASD. **(A)** Endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) are some of the targets for prenatal alcohol-induced oxidative stress in the neurovascular unit. **(B)** Superoxide levels at baseline in parietal cortex tissue from adult control rats, rats exposed to alcohol *in utero*, control + apocynin rats, control + rosiglitazone rats, alcohol + apocynin, and alcohol + rosiglitazone rats [Adapted and combined from 53 & 54 with proper permission from publisher, John Wiley and Sons]. *$p < 0.05$ *versus* levels before treatment with apocynin and rosiglitazone. **(C)** Proposed pathway for a physiological relationship between *in utero* alcohol exposure and impaired cerebral arterioles due to superoxide production. Expansion for NOX = NADPH oxidase.

baseline levels of superoxide in the parietal cortex tissue of rats (Figure 1B), even when NADPH, which was used to enhance NADPH oxidase activity, was administered. NOX-2 and NOX-4 are isoforms of the NADPH oxidase system that are found in endothelium and vascular smooth muscle and play a crucial role in the generation of superoxide [80–84]. Alcohol exposure during gestation increases the expression of NOX-2 and NOX-4 in microvessels. In addition, they discovered that apocynin, a powerful antioxidant, lowered superoxide levels (Figure 1C) and relieved impairment of eNOS- and nNOS-dependent responsiveness of cerebral arterioles in rats exposed to alcohol during gestation. This indicated an increase in superoxide generation in these animals, which may lead to an increase in oxidative stress in rats exposed to alcohol during gestation [52, 53]. Importantly, they discovered that apocynin reduces the likelihood of brain injury in adult rats after cerebral ischemia [53]. As apocynin was a nonspecific inhibitor of superoxide anion, the particular subcellular route underlying the impairment of cerebral vascular function remained unclear. Saha et al. used rosiglitazone, an agonist for the gamma subtype of peroxisome proliferator-activated receptors (PPARs) to determine the specific vascular anti-oxidant mechanism [54]. It has been shown that these receptors sit on vascular smooth muscle and endothelium [85,86,87]. In addition, both acute and chronic treatment of male and female adult rats with rosiglitazone protected decreased eNOS and nNOS-dependent vascular function in alcohol-exposed male and female adult rats. In addition, acute rosiglitazone decreased superoxide levels (Figure 1C) in parietal cortex tissue, indicating the anti-oxidant mechanism(s) *via* which

rosiglitazone enhanced vascular function [54]. PAE (three percent ethanol for entire gestation period) induced dysfunction in the ability of specific potassium channels to dilate in rat cerebral arterioles. This dysfunction appears to be mediated by an increase in oxidative stress, as acute apocynin was able to enhance the response [unpublished data].

Neurohormonal alteration

Vasoactive intestinal peptide (VIP) operates as a nonadrenergic, noncholinergic neurotransmitter or neuromodulator in both the peripheral and central nervous systems, where it acts on specific receptors to dilate cerebral arteries, pial arterioles, and intracerebral arterioles [88–91]. Alcohol exposure during gestation decreases VIP levels permanently in the rat fetal brain [92]. Alcohol exposure during pregnancy dramatically affected the dilator response of adult intracerebral arterioles to VIP in an adult sheep model of binge-drinking during pregnancy [50]. This decrease may suggest a loss of neuronal connections, including neurons carrying VIP, or a change in VIP receptor density in the adult brain. This is the first research to indicate that exposure to alcohol during fetal development may have long-lasting consequences on vasomotor responses in adult brain arteries.

Mechanism of modifications of morphology and integrity of NVC in response to prenatal alcohol exposure

PAE has profound impacts on angiogenesis and endothelial cell survival on the formation of cortical blood vessels. The molecular processes behind these effects are poorly understood. VEGF is an effective blood vessel development regulator. VEGF-R1, VEGF-R2, and VEGF-R3 receptors [93] mediate the biological actions of VEGFs. Jégou et al. [33] evaluated VEGF-R1 and VEGF-R2 protein levels in cerebral microvessel extracts of P2 mice exposed to prenatal alcohol in 2012. They observed an upregulation and also a simultaneous downregulation of VEGF1 and VEGF2 receptor proteins respectively in the cortical microvascular network during PAE; these changes were found to be associated with detrimental alterations in density and radial organization. Thus, disruption of these receptor subtypes is one of the mechanisms behind the VEGF-mediated modification of the cerebral microvascular network in rats prenatally exposed to alcohol. One of their *in vitro* experiments revealed that exogenous VEGF reduced the deleterious effect of ethanol on the vascular plasticity of the cortical glia [94].

During fetal development, well-controlled autophagy promotes vascular development and also protects against autophagic cell death. It is especially vital in ECs, one of the principal components of the developing blood vessels, as they help to adjust their bioenergetic and biosynthetic requirements in response to shifting environmental conditions, the presence of angiogenic stimuli, or intrinsic and extrinsic damages. PAE may inhibit autophagy in brain endothelial cells, hence leading to changes in angiogenesis and the resultant brain abnormalities identified in individuals with pFAS/FAS. PAE increases the frequency of autophagic vacuoles in the endothelium of cortical microvessels in human fetal brain tissues and in a mouse model of PAE in neonates, indicating defective autophagy [94, 95]. Girault et al. discovered that the levels of autophagy marker proteins, such as microtubule-associated protein 1A/1B-light chain 3 (LC3) LC3 and ubiquitin-binding protein p62, were considerably enhanced in endothelial cells treated with 50 mM ethanol in order to understand the process. In addition, a reduction in Rab7, a protein that plays a crucial function in endocytosis, was detected, which may account for the impaired autophagosome–lysosome fusion. Importantly, these effects of ethanol were eliminated in the presence of 4-methylpyrazole, which inhibits the synthesis of the ethanol metabolite acetaldehyde. These findings showed that acetaldehyde (MeCHO) triggers the process of autophagy dysregulation in cerebral microvessels after alcohol exposure [94].

In addition, it was shown that the increase in autophagy vacuoles after alcohol exposure was associated with a downregulation of the mammalian target of rapamycin (mTOR) pathway. They also demonstrated that activating autophagy with the mTOR inhibitor rapamycin reduces ethanol-induced endothelial cell death and restores vascular plasticity [94]. Overall, this shows that ethanol adversely affects angiogenesis by promoting endothelial autophagy in the cortical layer.

Previous research has shown that the neuroprotection afforded by autophagy may originate from the elimination of damaged mitochondria. And the lowered degree of autophagy may promote ROS production and excessive mitophagy [96], hence enhancing ethanol-induced cell death. In murine pulmonary microvascular endothelial cells (MPMVEC), Girault et al. found that ethanol impairs mitochondrial integrity and triggers apoptosis-inducing factor (AIF) protein release and nuclear translocation, which may result in programmed cell death. Therefore, they hypothesized that activation of autophagy by rapamycin may similarly shield endothelial cells from ethanol-induced mortality and aid cortical angiogenesis in individuals with pFAS/FAS [94].

Among numerous pathways of cell death generated by fetal brain alcohol exposure, fetal cerebral artery mitochondria-linked cellular apoptosis has been documented in an animal model of prenatal alcohol exposure and FASD-related brain injury [97]. Alcohol-induced abnormalities in prenatal cerebrovascular mitochondria may result from both direct targeting by alcohol [29, 98] and secondary damage resulting from alcohol-induced

changes in fetal cerebral blood flow [41, 46]. Therefore, mitochondria-targeted therapies with ROS scavengers and antioxidants might be a viable therapeutic strategy for the treatment of FAS/FASDs.

The integrity of cortical microvessels is necessary for optimal vascular development. Permeability of the BBB and matrix metalloproteinases (MMP)-induced proteolysis of the neurovascular matrix may influence cortical vascular development. BBB permeability has also been proposed as a possible site of PAE-induced change in cerebral capillaries. However, no documented evidence of prenatal ethanol-induced BBB permeability currently exists. In contrast, MMP-induced proteolysis of the neurovascular matrix may also cause programmed cell death by cell separation from the extracellular matrix [99, 100]. Indeed, glutamate-induced activation of the endothelium protease MMP-9 from pial microvessels of neonates was seen in a mouse model of PAE [101].

As yet, no global mechanism of alcohol-induced impairment to embryonic or fetal brain development has been revealed, and it is very unlikely that a single mechanism can explain the various components of the FASD presentation. In addition, while alcohol is often regarded the principal chemical that causes birth defects (i.e., a teratogen), alcohol's breakdown products (i.e., its metabolism) may also play a role. For instance, acetaldehyde, a toxin produced by the breakdown of alcohol in the liver and other organs, may accumulate in the fetal brain during prenatal alcohol exposure and may contribute to the development of FASD. Each individual shows a unique mix of alcohol-related consequences, which is influenced by the time, amount, pattern, and length of the mother's drinking, in addition to hereditary variables. This variation makes it difficult to compare the effects of drinking across individuals.

Conclusion

There is currently no readily available treatment for intentional or unintentional alcohol intake during pregnancy. The inadequate mechanistic knowledge of FASD's pathogenesis is one of the explanations. More research is required to understand the underlying mechanisms through which alcohol might affect the structure and function of cells. Understanding these multiple mechanisms and seeking to inhibit them may help us to reduce the negative effects of alcohol exposure on embryonic development in the future. Additionally, efforts must be made to improve public awareness of the detrimental consequences of even little alcohol intake during pregnancy.

Author contributions

Study conception and design: PS and WM; Draft manuscript preparation: PS. All authors reviewed the contents and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Common developmental trajectories and clinical identification of children with fetal alcohol spectrum disorders: A synthesis of the literature

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At an estimated prevalence of up to five percent in the general population, fetal alcohol spectrum disorders (FASD) are the most common neurodevelopmental disorder, at least if not more prevalent than autism (2.3%). Despite this prevalence in the general population, pediatricians and other developmental specialists have thus far failed to diagnose this disability, leaving most children and adults without the supports provided for most other disabilities. This paper will provide a review of clinically relevant literature that describes the developmental challenges of children with fetal alcohol spectrum disorders and addresses similarities to and differences of FASD from other neurodevelopmental disorders such as autism and attention deficit hyperactivity disorder. A subsequent discussion will describe how a diagnosis of an FASD can establish a basis for understanding the developmental and behavioral challenges of children with an FASD, and how specific interventions can help support child development and maximize adult independence.

KEYWORDS

prenatal alcohol exposure, fetal alcohol syndrome, fetal alcohol spectrum disorders, neurodevelopmental disorder associated with prenatal alcohol exposure, prenatal substance exposure, foster care

Introduction: The prevalence of children with fetal alcohol spectrum disorders

In the 50 years since the effects of prenatal alcohol exposure upon fetal development were first described as a constellation of facial features, growth impairment, and neurodevelopmental impairments designated fetal alcohol syndrome (1), the effects of alcohol upon prenatal brain development and its subsequent neurodevelopmental

Abbreviations: ADHD, attention deficit-hyperactivity disorder; ASD, autism spectrum disorder; FASD, fetal alcohol spectrum disorders; FAS, fetal alcohol syndrome; ND-PAE, neurodevelopmental disorder associated with prenatal alcohol exposure; ODD, oppositional defiant disorder; PAE, prenatal alcohol exposure.

sequelae have been expanded to include developmental challenges even in the absence of facial features and/or growth impairment associated with fetal alcohol syndrome. This broader category are the fetal alcohol spectrum disorders (FASD).

Fetal alcohol spectrum disorders are so common that a physician can be certain he or she has cared for a child with this disorder. Physicians can be just as certain that no professional previously diagnosed that child with an FASD (unless they were the person who suspected this diagnosis). Despite widespread warnings, women often receive conflicting messages from professionals on the safety of alcohol use during pregnancy and alcohol use during pregnancy continues to be prevalent. A recent CDC study found that 13.5% of pregnant adults reported current drinking and 5.2% reported binge drinking in the past 30 days (2). Estimates of alcohol consumption by non-pregnant women of child-bearing age (18–44 years) range from 53% of any alcohol use to 18.2% of women who binge drink with the majority of women being college-educated and employed (3). This statistic becomes especially important since many women do not discover their pregnancy until after missing their regular menstruation at 4–6 weeks gestational age, during which time brain development has already been effected by neurotoxic exposure. Common reasons for alcohol use in pregnancy are lack of awareness of the adverse effects of alcohol upon the fetus, the belief that only excessive alcohol use is harmful, maternal stress during pregnancy, unwanted or unplanned pregnancy, and alcohol dependence (4).

A study of first grade children in schools across four sites in the Midwest United States, found a total prevalence of FASD of 1.1%–5% (up to one in twenty children) (5). While the span in prevalence estimates likely reflects regional variation across the US in alcohol use during pregnancy, this figure highlights that FASD is a disorder as common as any other medical condition physicians diagnose and treat each day. The US Census Bureau estimates that in 2020, there were 72.8 million children living in the United States[1], making the range of the number of children with FASD between 0.8 and 3.64 million children based upon FASD prevalence documented above. Yet compared to asthma, a disease with an estimated prevalence of 1 in 12 children (8.3%) (6) or the 1 in 44 children with autism (2.3%) (7), physicians and other professionals rarely consider FASD among their differential diagnosis as a cause of developmental and behavioral challenges.

FASD is even more prevalent among children in foster care, where an estimated 16.9% of children are affected by an FASD (8). Yet a diagnostic clinic evaluating children referred from foster care for developmental and behavioral challenges, found 80% of children who were subsequently diagnosed with an FASD, had never been previously identified with this disorder (9). Parental substance and alcohol use disorders are one of the most common reasons for foster care placement (10). Screening of all children entering child welfare for prenatal exposure to alcohol and other substances is far from routine. Even when prenatal exposure to other substances such as cannabis, cocaine, or opioids is documented in newborn medical records, screening for prenatal alcohol exposure is notably absent in most obstetric, pediatric, and child welfare records (11, 12).

Current clinical guidelines for diagnosis and assessment state that "assignment of an FASD diagnosis is a complex medical diagnostic process best accomplished through a structured multidisciplinary approach by a clinical team comprising members with varied but complementary experience, qualifications, and skills" (13). But multiple and often discrepant classification systems and standards of what constitutes a diagnosis of FASD in the United States create a major barrier to diagnosis because of the lack of uniform language to define FASD. Countries such as Canada and Australia have adopted uniform diagnostic criteria that allow a nation-wide, consistent approach to diagnosis with detailed clinical practice guidelines to support assessment, treatment, and disability qualification (14,15). Persistent recommendations for a comprehensive, multidisciplinary assessment might be appropriate if such resources were readily available in the United States. However, given the prevalence of FASD and the scarcity of diagnostic resources in the United States, how can children with an FASD be more readily identified and treated? Can children with an FASD be differentiated from children with other neurodevelopmental disorders? Is there sufficient consensus in the literature to suggest a diagnostic path that can allow physicians and other professionals a means to provisionally identify children with FASD in the absence of a multidisciplinary team and initiate treatment recommendations?

This article reviews common developmental trajectories of the neurodevelopmental disorders, including autism, global developmental delay, speech delay, ADHD, intellectual disability, and FASD. After reviewing similarities and differences across the neurodevelopmental disorders and a process for screening for prenatal alcohol exposure, the criteria of neurodevelopmental disorder associated with prenatal alcohol exposure is described as a pathway for practitioners to begin identifying and treating children with suspected FASD.

Methodology

Literature searches were completed through PubMed for all articles utilizing the search terms of "prenatal alcohol exposure" crossed with "developmental trajectory" and "diagnosis" for the years 2010–2022 in all languages yielded a total of 21 results in PubMed. A more restricted search limited to review articles using the terms "neurodevelopmental disorders crossed with

1 https://www.childstats.gov/americaschildren/tables/pop1.asp (Accessed 26 July, 2022).

"developmental trajectory" and "diagnosis" for the years 2010–2022 in all languages yielded a total of 773 articles. A total number of 794 articles were subsequently reviewed by title and abstract for relevance to the topic of this article (Figure 1).

These results, including citations and references, were reviewed based upon their relevance to the similarities and differences of development among children with an FASD compared to other neurodevelopmental disorders. Additional focused searches were made based upon the need for supporting documentation during the writing of the article.

Results

A structured clinical approach to diagnosis and intervention was synthesized based upon the diagnostic criteria for neurodevelopmental disorder associated with prenatal alcohol exposure (16,17) to help clarify a clinical means of identifying and initiating interventions for children with suspected FASD in general pediatric practice and during other developmental assessments.

Common neurodevelopmental disorders and their developmental trajectories

The category of neurodevelopmental disorders spans developmental challenges that present during early childhood as manifestations of manifold and yet to be clearly defined differences in brain development. Estimated prevalence rates for the neurodevelopmental disorders range from 0.63% to 3%

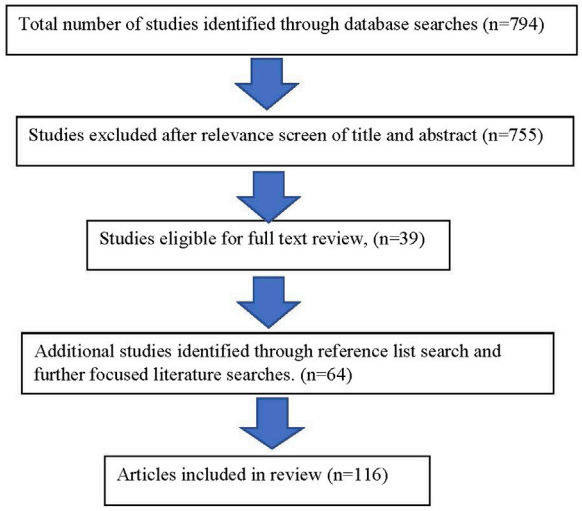
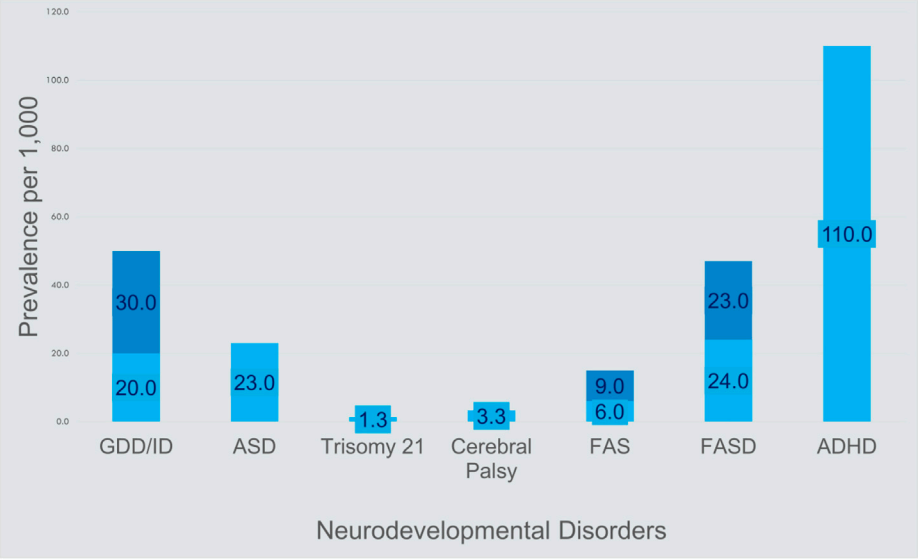


FIGURE 1
Depiction of methodological search of literature.

for intellectual disability, 5%–11% for ADHD, 3%–10% for specific learning disorders, 42% for communication disorders, and 0.76%–17% for motor disorders (18). Causes of neurodevelopmental disorders range from genetic abnormalities, pre- or post-natal infections, asphyxia, prematurity, epigenetic changes prior to or after birth, prenatal or post-natal exposure to neurotoxins, nutritional deficiencies, and prenatal fetal or maternal medical conditions including maternal mental health (19). The interplay across this list is complex and captured in the relatively new fields of epigenetics and the neuroendocrine immune system (20). The result is manifested as disabilities such as global developmental delay, intellectual disability, autism, attention deficit disorders, learning disabilities, speech/language disorders, and fetal alcohol spectrum disorders. Figure 2 shows the lower and upper estimates for the primary neurodevelopmental disorders discussed in this article compared to more commonly known specific disabilities of Trisomy 21 and cerebral palsy.

The pretense of these terms becomes apparent in clinical practice as these disorders manifest many common characteristics and often appear simultaneously with a great overlap of developmental challenges. The wide prevalence estimates given above for each neurodevelopmental disorder further hint at the great overlap among these diagnoses (Figure 2). This overlap leads inevitably to the question of whether the neurodevelopmental disorders are distinct disorders. Comorbidity among the neurodevelopmental disorders is the rule rather than the exception and the spectrum of these developmental challenges exists along a continuum of severity (26). In addition, developmental challenges often shift and become more differentiated over time leading to a clearer, more specific diagnosis with advancing chronological age. In clinical practice, the question becomes to what extent a specific diagnosis provides a context for a discussion of a child's behavioral challenges with the family and a starting point to obtain intervention services (27).

The primary challenge in assessing a child with developmental delay becomes one of differentiating a child with autism, FASD, or global developmental delay, from a child with isolated speech delay (communication disorder). Diagnosis often relies upon the pattern of developmental challenges as measured across developmental domains on standardized tests that document function outside of the normal range of development (28). The lack of specific biological markers and lack of specific distinct etiologies makes developmental assessment more challenging and subjective despite the use of standardized developmental tests. Nevertheless, the concept of a neurodevelopmental disorder as developmental delays that present over the course of development is useful in seeking to identify children who require intervention, gives a language to communicate with parents and other professionals, and provides a pathway for obtaining community-based services. A detailed history that focuses upon the pattern of a child's early developmental trajectories across



Abbreviations: GDD/ID=global developmental delay/intellectual disability; ASD=autism spectrum disorders; FAS=fetal alcohol syndrome; FASD=fetal alcohol spectrum disorders; ADHD=attention deficit hyperactivity disorder

FIGURE 2
Prevalence of common causes of disability (21–25)[2]. Abbreviations: GDD/ID, global developmental delay/intellectual disability; ASD, autism spectrum disorders; FAS, fetal alcohol syndrome; FASD, fetal alcohol spectrum disorders; ADHD, attention deficit hyperactivity disorder.

domains of developments forms the basis for distinguishing the neurodevelopmental disorders to facilitate diagnosis and intervention. Prior to reviewing the spectrum of developmental delays found in the neurodevelopmental disorders, this article will briefly review the trajectory of normal development from birth to age 3 years.

Because the diagnosis criteria for ADHD which include impairments in attention and self-regulation (hyperactivity/impulsivity) are often present across most neurodevelopmental disorders, I will focus on this neurodevelopmental disorder last as a diagnosis of exclusion of the four core neurodevelopmental disorders: fetal alcohol spectrum disorders, autism, global developmental delay, and speech/language disorders.[2]

A simplified clinical approach to developmental assessment

Gessell noted that normal development proceeds in an orderly, timed, and sequential process that occurs with such regularity that it is predictable (29). While there is variation from child to child within the framework of normal development, a common normal trajectory is depicted below in Figure 3 (30). The departure from expected developmental trajectories helps to identify children needing assessment. Current recommendations by the American Academy of Pediatrics recommend developmental screening of all children using a validated developmental screening test at the 9-, 18-, and 30-month visits. This recommendation aids in identification of children at risk of developmental delays and autism. Screens typically include the Ages to Stages Questionnaires and Modified Checklist for Autism (31).

Evaluation for speech delay is the most common cause of referral for developmental evaluation. The initial task in evaluating speech delay is determining whether this is a case of isolated speech delay (communication disorder), or a neurodevelopmental disorder that spans other domains of development such as autism, global developmental delay, and FASD.

In obtaining a developmental history, initial queries can focus on the temperament of a child including patterns of sleep, ability to be soothed, and activity level. Infant temperament is associated with attachment which is the basis for gains in social interactions and subsequent acquisition of speech and language (32, 33). Absence of eye contact and reactive smile in the first months of infancy is often one of the first signs of autism (34, 35). Eye contact and social engagement lead to

2 https://www.cdc.gov/ncbddd/adhd/data.html (Accessed 15 January, 2023).

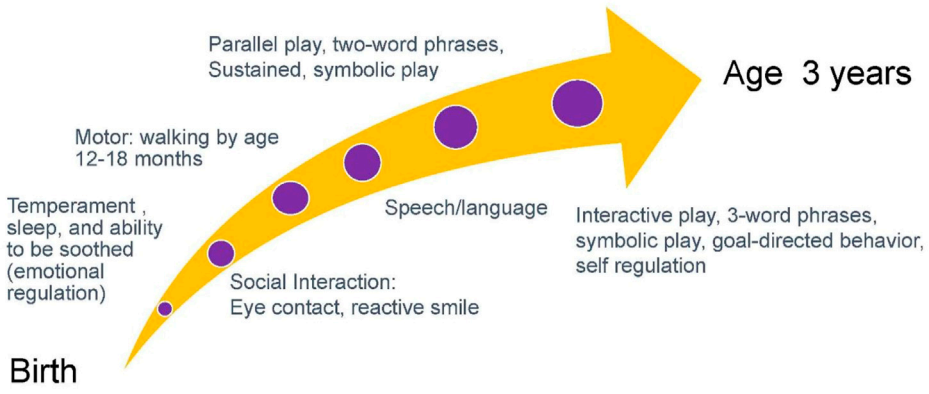


FIGURE 3
Milestones of normal development.

reactive cooing and the first stages of verbal interaction that subsequently progress to use of repetitive syllables (babbling) between 6 and 9 months and intelligible speech around age 1 year (36). Motor milestones proceed in a cephalo-caudal direction with progression from gaining head control by 3 months, to lifting chest when prone by 5 months, to sitting at 6 months, crawling at 9 months, and walking by 1 year. The development of speech and ambulation allows the infant to explore the world and engage more readily in interactions with others. Social development includes eye contact, use of non-verbal gestures such as indicative pointing, and motivation to seek out social interactions. These processes are concurrent with increased attention and cognitive ability that allow the acquisition of sustained and symbolic play by age 2 years. While children around age 2 years tend to engage in parallel play (minimally interactive play in proximity to other children), by age 3 years children engage in interactive play and goal-directed behavior (37). Concurrent with this development is the emerging ability to regulate emotions such as frustration that facilitates social interactions (38). Children with neurodevelopmental disorders frequently present with variable patterns of delays in development across developmental domains. Figure 4 charts an example of the developmental trajectory of a child with a neurodevelopmental disorder. This deviation from expected patterns of development allows practitioners to identify a constellation of developmental challenges that helps establish a differential diagnosis.

Children with global developmental delay present the clearest example of altered trajectories of development. Pervasive delays across two or more developmental domains of cognitive, adaptive, social-emotional, gross and fine motor, and speech domains characterize the challenges of children with global developmental delay (39). The diagnosis of global developmental delay is limited to children under the age of 5 years who are unable to undergo systematic assessments of intellectual functioning, including children who are too young to participate in standardized testing (16,40). While use of standardized testing measures such as those employed in evaluation by early intervention and schools can verify clinical suspicion, cognitive ability in children younger than 3 years is in flux and isolated testing is often unreliable (41). A common clinical practice is the use of the developmental quotient obtained by dividing the child's estimated developmental age over their current chronological age. Developmental quotients below 70 strongly suggest delays in that specific domain. While intellectual disability may later be diagnosed in a child with global developmental delay (the prevalence of global developmental delay, like that of intellectual disability, is estimated to be 1% to 3%) (21), not all children with global delays will go on to meet criteria for an intellectual disability (42).

Overall prevalence rates for specific language impairment in kindergarten children is estimated to be 7.4% with higher prevalence for boys (8%), compared to girls (6%) (43). A study of 7,267 children aged 4–5 years found the estimated prevalence of language disorder of unknown origin to be 7.58% while the prevalence of language impairment associated with intellectual disability and/or other medical diagnosis was 2.34% (44). While children with language disorder may have greater symptoms of social, emotional, and behavioral challenges compared to peers and often have later challenges in school achievement, they lack the major delays in the other developmental domains of socio-emotional, adaptive, and cognitive ability and the repetitive behaviors and lack of social

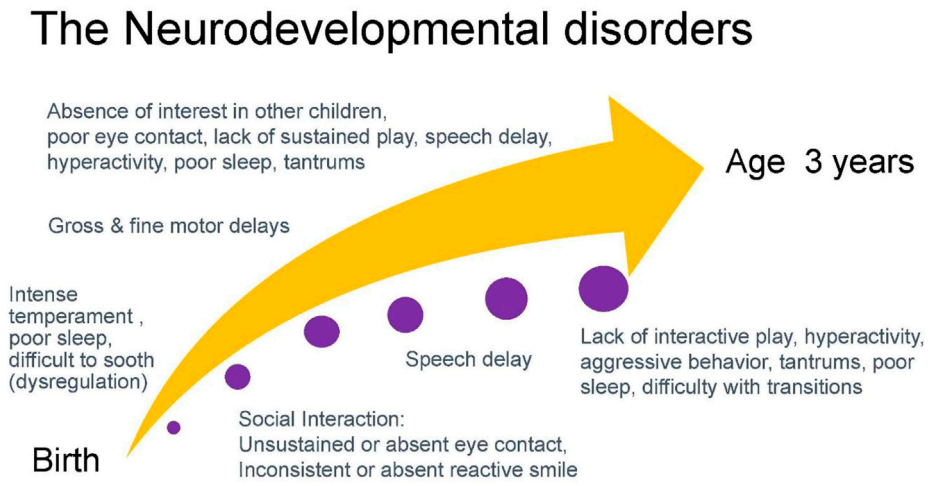


FIGURE 4
An example of a common developmental trajectory of a child with a neurodevelopmental disorder.

interest found among children with autism. Thus, in assessing a child with speech delay, concurrent delays in other domains of development can help differentiate the primary neurodevelopmental disorders (45). An audiologist should evaluate all children with speech delay to rule out conductive or sensorineural hearing loss.

In contrast to the global delays or isolated speech delays described above, a diagnosis of autism rests upon impairments in social and communication domains along with signs of restricted interests and repetitive behaviors (16). These include impairments in social-emotional interaction such as eye contact, lack of socially reactive smile or emotion, and lack of interest in initiating or responding to social interactions. Restricted, repetitive patterns of behavior, interests, or activities can be seen as repetitive motor movements, use of objects, or speech such as pacing, spinning, repetitive hand-eye movements, and echolalia. Insistence on sameness and rigid routines leads to severe emotional dysregulation, and highly restricted, fixated interests that are abnormal in intensity or focus (strong attachment to single objects), as well as hyper- or hypo-reactivity to sensory input (sounds, textures, smell, touch, visual fixation on details or lights or movement) (46).

It is therefore unsurprising that up to 62.3% of children with global developmental delay also meet diagnostic criteria for autism (47). In some cases regression of development occurs after the first year of life, prior to which socio-communicative skills might have appeared normal to parents (48). Beyond neurodegenerative disorders such as Rett syndrome, few other neurodevelopmental disorders present with the regressive loss of communication or social interaction described by parents of children with autism. Broad estimates across studies suggest 11%–65% of school-age children with autism subsequently also have the additional diagnosis of intellectual disability (49). Children with autism or global developmental delay often have severe behavioral challenges, the severity of which inversely correlates with the child's developmental quotient and cognitive ability (50). Symptoms of inattention and hyperactivity can easily be diagnosed as ADHD without recognition of an underlying diagnosis of autism or intellectual disability. Genetic evaluation and testing should be considered in all children with suspected autism, global developmental delay, and intellectual disability as well as children with suspected FASD to help exclude genetic causes of developmental challenges which can be present in addition to prenatal alcohol exposure.

Neurodevelopmental disorder associated with prenatal alcohol exposure

A path to increase identification of children with FASD

Given the breadth and overlap of the three primary neurodevelopmental disorders highlighted above, how can pediatricians begin to discern children with an FASD from other neurodevelopmental disorders? How can practitioners identify and establish interventions for children living with an FASD in the absence of an FASD multidisciplinary diagnostic center? The DSM-5 diagnostic criteria for neurodevelopmental disorder associated with prenatal alcohol exposure (ND-PAE) provides a straightforward path for practitioners to establish a provisional diagnosis much as pediatricians currently identify children with suspected autism (see Figure 5). One or more impairments in neurocognitive function, one or more impairments in self-regulation, and two or more deficits in

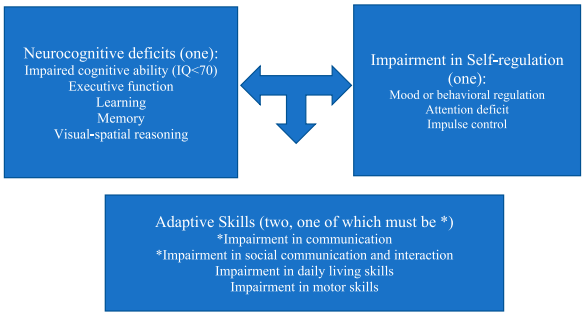


FIGURE 5
Criteria for neurodevelopmental disorder associated with prenatal alcohol exposure.

adaptive function (with at least one being one of the first two symptoms highlighted by an asterisk) are sufficient to establish a diagnosis of ND-PAE if there is confirmed history of prenatal alcohol exposure (51).

Since its inclusion in the DSM-5 as a "condition for further study," there appears to be strong correlation between the diagnostic categories of FASD and ND-PAE (52, 53). The advantage of ND-PAE criteria however is the emphasis on neurodevelopmental manifestations that practitioners see daily without extensive focus upon facial features and growth impairment that are often a barrier to diagnosis. While the clinical diagnostic guidelines for a diagnosis of ND-PAE may be less sensitive and specific than the traditional standards for the diagnosis of FASD, the utility of ND-PAE guidelines for front-line practitioners make this a step toward identifying children with an FASD (54).

A diagnosis of ND-PAE requires:

(1) One or more neurocognitive deficits
(2) One or more impairments in self-regulation
(3) Two or more impairments in adaptive skills, one of which must be communication deficit or impairment in social communication and interaction
(4) Documentation of more than minimal prenatal alcohol exposure

Children with FASD range in presentation from global developmental delay, symptoms of autism, isolated speech delay, or isolated early behavioral challenges similar to those seen among children with ADHD (Table 1) (55). Despite the great overlap across the neurodevelopmental disorders, differences in developmental challenges become more apparent with increasing chronological age. For example, young children with FASD may be diagnosed with autism because of speech delay, difficulty with transitions, sensory processing issues, socially inappropriate behaviors, difficulties with interpersonal interactions, and emotional/behavioral dysregulation. Challenges in social cognition are a primary challenge among children with FASD. Individuals with FASD have greater difficulties interpreting facial emotions than typically developing children (56). But while children with FASD share challenges in social skills and behavioral issues that can lead to a diagnosis of autism, children with FASD often score low in repetitive behaviors and restricted interests (57). Table 1 highlights common developmental challenges of children with FASD between ages 4–12 years.

TABLE 1 Middle school developmental challenges of children with FASD.

Developmental challenges of children with FASD: Ages 4–12 years
• Difficulties with receptive language compared to expressive language (auditory processing); Difficulties with conversation
• Difficulties in peer interactions (reading non-verbal cues, auditory processing, emotional/behavioral dysregulation, inappropriate interpersonal boundaries)
• Hyperactive, poor attention, disorganized (often referred for ADHD evaluation by age 3–4 years)
• Impulsivity, lack of awareness of danger and consequences
• Learning challenges (learns it then forgets it)
• Difficulties with tasks of daily living ("you should be able to do this at your age")
• Confabulation
• Aggressive behavior
• High risk for school suspension or expulsion (as early as kindergarten)
• Sleep difficulties

Yet more subtle differences can distinguish FASD from autism. The difficulties in initiating social interaction, sharing affect, and using non-verbal communication common in children with autism are less common in children with FASD who tend to seek out social interaction at the exclusion of awareness of interpersonal boundaries. Similarly, while children with ASD are often referred to as aloof or uninterested in social interaction, children with FASD are more likely to make sustained eye contact, use indicative pointing to show or express interest or direct attention (theory of mind), engage in social interaction (often with an overly social and indiscriminately friendly presence), engage in interactive play and simple conversation, and offer comfort to others (58). Children with FASD are often overly friendly and lack interpersonal boundaries (as opposed to preference for solitary play). They are also at higher risk for behavioral challenges with symptoms of hyperactivity, impulsivity, and aggression easily mistaken for self-willed behaviors and attributed to a psychiatric disorder (ADHD) or an "emotional disturbance" instead of as a manifestation of neurological impairment. These challenging behaviors are the result of impairments in brain function across memory, learning, cognitive flexibility, comprehension, attention, planning, social skill development, and learning (59, 60). School evaluations often

view learning challenges among children with an FASD as simple issues with attention and motivation, rather than as an underlying static encephalopathy or learning disorder. Because most children with an FASD are frequently not diagnosed with this disorder, their behaviors place them at higher risk for school suspension or school failure.

As noted at the beginning of this article, the prevalence of ADHD ranges from 5%–11% in the general population. ADHD is a neurodevelopmental disorder defined by impaired levels of inattention, disorganization, and/or hyperactivity-impulsivity. These are manifested by inability to stay on task, seeming not to listen, losing materials, being overly active, inability to stay seated or wait, and intruding into other people's activities at levels that are excessive and inconsistent with age or developmental level (16). ADHD is commonly diagnosed among children with autism, global developmental delay, intellectual disability, and fetal alcohol spectrum disorder. At least 41%–48% of children with prenatal alcohol exposure are diagnosed with ADHD (61,62). Clinical experience suggests this percentage is far higher but limited by lack of screening for prenatal alcohol exposure in ADHD prevalence studies (63).

Children with FASD and ADHD both have challenges in executive function, including working memory, attention, behavioral regulation, and impulse control. Early challenges in executive function (the ability to focus attention, engage in sustained play, have goal-oriented behavior, and regulate emotions across different environments) and social function (the ability to engage in joint attention, exhibit social reciprocity and sharing, and perspective taking) are common to preschool children with FASD and become more apparent with age (64). Marked behavioral problems often eclipse the neurological impairments of FASD, commonly leading to multiple psychiatric diagnoses (ADHD, ODD, conduct disorder being the most common). Thus, behavioral referral of children with undiagnosed FASD nearly always leads to a diagnosis of ADHD in early childhood. The more recent concept of complex ADHD describes children with early onset of ADHD before age 4 years and have moderate to severe functional impairment, or inadequate response to treatment (65). Worsening of behavior or failure to respond to stimulant medication or lack of response to typical behavioral interventions should suggest a possible underlying diagnosis of FASD among children with a diagnosis of ADHD (66). While research is needed to clarify the extent of prenatal alcohol or other substance exposure among children with complex ADHD, the severe early developmental trajectory is strongly similar to those seen with FASD.

FASD is the most common identifiable cause of secondary morbidities such as intellectual disability, ADHD, anxiety disorders, and learning disabilities (63, 67). Children with multiple psychiatric diagnoses such as ADHD, oppositional deficient disorder, disruptive mood dysregulation disorder, conduct disorder, or intellectual disability should be screened for prenatal alcohol exposure to ensure a diagnosis of FASD is not the underlying cause of the severe emotional/behavioral dysregulation that attends each of these disorders (67). Furthermore, challenges in shifting activities and difficulties with transitions are common among children with autism and often a primary behavioral challenge of children with FASD, resulting in emotional/behavioral dysregulation during transitions or limit-setting (60). Children with FASD typically require a highly structured environment and become more dysregulated in overstimulating environments (68). The poor ability of many children with FASD to regulate sensory stimulation mirrors the challenges of children with autism who easily become dysregulated by sensory overstimulation. This may help explain why children with FASD often fail to respond well to stimulant medication but are more responsive to medication that targets emotional/behavioral dysregulation and symptoms of anxiety (69)[3].

Identifying impairments in adaptive function is critical to understanding the developmental challenges of children with FASD (Figure 6). Adaptive function is the ability to complete day-to-day age-appropriate tasks and spans skills such as receptive and expressive communication, social interaction, coping skills, and activities of daily living. While children with FASD, autism, intellectual disability and ADHD often show adaptive behavior impairments, the relationship of cognitive ability to adaptive function often helps distinguish children with FASD from the other neurodevelopmental disorders. The DSM-V defines intellectual disability as impairments in both cognitive and adaptive function. In contrast, children with autism without intellectual disability typically score low in the communication and socialization domains of adaptive function and often present with a significant gap between high non-verbal and low verbal abilities on cognitive testing (70). Estimates of cognitive ability among children with FASD vary widely, ranging from 20 to 120, with an average of about 72. Children with FASD typically have significantly lower IQ scores than those with ADHD and score lower in adaptive functioning when compared to IQ matched children (71). While adaptive skills improve with age for children with ADHD, children with FASD tend to be significantly more impaired in the daily living skills domain and impairments in both socialization and communication domains become more apparent with age (Figure 6) (72, 73).

Individuals with FASD often have normal to borderline cognitive ability (above 70) and frequently fail to meet criteria for intellectual disability or autism leading to assumptions that they can complete tasks "if only they try harder." Coles et al. have described two subsets of children with alcohol-related neurodevelopmental disorder: one with cognitive

3 https://canfasd.ca/algorithm/ (Accessed 16 January, 2023).

impairment, the other with primarily behavioral manifestations. Of note, both children with primarily behavioral manifestations and those with cognitive impairments both scored low on adaptive function (74). The Collaborative Initiative on Fetal Alcohol Spectrum Disorders has proposed a decision tree to help identify children with PAE, but these criteria rely on psychometric measures such as the Child Behavioral Checklist and the Vineland Adaptive Behavioral Scale that are often unavailable to practitioners without access to neuropsychological testing (75).

Evaluations by the school can fail to identify impaired adaptive function in the face of normal to low cognitive ability. This "intellectual disability equivalence" often leads to difficulties in learning, social interactions, and behavior that become increasingly apparent as children move through adolescence (76) (see Figure 6). The inability to qualify for services available to individuals with autism and intellectual disability leaves a gap in services that is one of the greatest struggles for families caring for children with an FASD. The failure to identify challenges in adaptive function and the subsequent blame placed upon children from early childhood to adulthood for difficulties with basic age-appropriate tasks not only damages self-esteem, but over time leads to anxiety, depression, and even suicide in individuals living with an FASD.

Barriers to FASD diagnosis include lack of awareness of FASD prevalence, manifestations, and diagnostic criteria and discomfort of professionals in discussing prenatal exposures. In addition, there is a lack of systematic screening for prenatal alcohol exposure by obstetricians, pediatricians, psychiatrists, psychologists, and social workers; lack of a biological marker for diagnosis of FASD; and underreporting of alcohol use during pregnancy due to stigma and fear of repercussions (77). Early recognition of exposure allows risk stratification to identify children who need closer developmental follow up. Stigma against women with substance and alcohol use disorders continues to be a barrier to diagnosis, especially in the child welfare system where parents feel judged and threatened with termination of their parental rights. Each of these barriers leads to a lack of resources for interventions services specific to an FASD diagnosis. The sum of these failures leads to a need for constant advocacy by providers and families in gaining services for children whose neurological impairments frequently manifest as behavioral and psychiatric challenges.

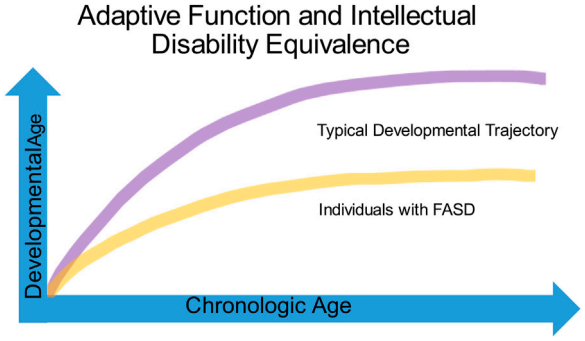


FIGURE 6
Adaptive function and intellectual disability equivalence.

Screening for prenatal alcohol

When evaluating a child with developmental or behavioral challenges, screening for prenatal alcohol exposure (PAE) is the single most important first step in considering a diagnosis of FASD. Prenatal alcohol exposure alone can be a predictor of child development. A study comparing documented prenatal exposure using the biological marker, meconium ethyl glucuronide, and cognitive deficits and symptoms of ADHD, found a partially dose-dependent relationship to development (78). Therefore, a brief review of a process for obtaining a history of prenatal exposure deserves discussion.

Evaluation can easily incorporate screening as part of obtaining the prenatal and birth history that is routine for most practitioners. The effectiveness of weaving questions for PAE into the prenatal history makes asking questions that are often uncomfortable for both professionals and parents, easier to present as a routine part of information gathering. Below is a simple script practitioners can complete quickly in even the busiest of practices.

- How far into your pregnancy did you discover you were pregnant?
- Did you have any medical problems during your pregnancy?
- Were you prescribed any medications during your pregnancy?
- How much alcohol did you use prior to finding out you were pregnant?
- How much alcohol did you use after finding out you were pregnant?
- What other substances did you use before and after you found out you were pregnant (such as cannabis, opioids, or other non-prescribed medications)?

Note the importance of obtaining a history of alcohol and other substance exposure prior to pregnancy recognition. After obtaining a positive history of alcohol use prior to or after pregnancy recognition, further investigation of alcohol preference (beer, wine, liquor) and the size of a typical drink helps clarify the extent of alcohol-related neurotoxic exposure.

Suggested guidelines for significant alcohol exposure have been made (79), but current evidence documents that even small amounts of prenatal alcohol exposure can affect brain development and that there is no known "safe" amount of alcohol use in pregnancy (80). While there is no safe amount of alcohol consumption during pregnancy, binge drinking with sharp elevated maternal blood alcohol levels readily cross the placenta and carry the highest risk for a fetal alcohol spectrum disorder. Greater maternal blood alcohol levels are associated with greater severity within the spectrum of FASD, with higher levels associated with fetal alcohol syndrome (81). In addition to a direct maternal interview, information of alcohol consumption during pregnancy can also be obtained from a reliable collateral source such as a family member, social service agency, or prenatal or maternal medical records. Definitions of significant alcohol exposure vary, but consumption of six or more drinks per week for more than 2 weeks or three or more drinks on two or more occasions can be considered a guideline for determining significant alcohol consumption (82). Additional history of significant alcohol consumption can also be obtained from documentation of social or legal problems associated with alcohol use or intoxication during pregnancy (83).

Screening for PAE is often an iterative process that may require revisitation and in which a parent who may initially deny use of alcohol during pregnancy, later discloses use in the context of a relationship of trust that focuses upon the wellbeing of the child. Even with documented prenatal alcohol exposure, a diagnosis of an FASD is inappropriate until further psychological standardized testing (including testing for adaptive function) and evaluations by early intervention or the school can be completed. The discussion of a diagnosis of FASD with a parent requires patience, frequently starting with interventions before clarifying the suspected etiology of developmental delays. Anticipatory guidance of potential developmental and behavioral challenges and the possible need for additional support in the future builds a working relationship with parents and diminishes the helplessness, guilt, and feeling of aloneness that comes with caring for a child with severe developmental and behavioral difficulties.

Interventions: Bending the trajectory

The primary reason for any diagnosis is intervention. Diagnosis allows education of caregivers about the disabilities and anticipatory guidance for risks and current or future need for interventions. Diagnosis also allows individuals with FASD to better understand their strengths and weaknesses ("blind spots"). Ideally diagnosis also allows access to disability services. In most cases the subjectivity of a diagnosis rests upon clinical experience and awareness of the importance of diagnosis in obtaining services. Diagnosis also allows a common language for clinicians to discuss developmental challenges in the context of a diagnosis, including framing a prognosis, and providing anticipatory guidance for possible future challenges. Table 2 highlights common developmental challenges of children as they move through adolescence.

Vygotsky's model of the zone of proximal developmental provides a framework for helping parents and teachers greater awareness of developmental challenges and providing services that meet the child at the level of their developmental ability (Figure 7) (84–86). The zone of proximal development is based upon the three zones of the ability of the child to complete tasks without help or guidance, with support and guidance, and the level beyond which the child is unable to complete a task even with adult support. Identifying these impairments helps guide interventions that meet a child's level of function and is imperative in working with all children with disabilities.

While children with neurodevelopmental disorders such as speech delay, autism, and intellectual disability have a clear pathway to services, families of children with an FASD often find providers who lack training in caring for children with an FASD. There is an urgent need to establish a community network of service providers familiar with the challenges and interventions to support the development of children with an FASD (Figure 8). This network of services would ideally begin with early identification of children with an FASD prior to age 3 and include early intervention providers familiar with the challenges that attend a diagnosis of FASD. It would include family support services across childhood while ensuring transition from early intervention services to special education services and beyond (87). A major barrier to supportive services for families of a child with an FASD is the lack of inclusion of FASD as a developmental disability eligible for state-based disability services under the Individuals with Disabilities Act. While many states allow eligibility for disability services for

TABLE 2 Developmental challenges of adolescents and young adults with FASD.

Developmental challenges of adolescents with an FASD: Ages 13–21 years
• Increasing gap between chronological age and developmental age, especially in adaptive function (tasks of daily living, maintaining safety, difficulties with managing time and money), "18 going on 10"
• Difficulties with respective language (auditory processing), reading non-verbal social cues engaging in back-and-forth conversation
• Difficulties making and keeping friends
• Poor interpersonal boundaries, sexually inappropriate behavior
• Gullibility, easily swayed by others to do acts they would not do alone
• Confabulation, taking possessions of others, stealing
• High risk for school failure/drop out
• Parent-child relationship difficulties including increasing use of aggression and destructive behavior in the home
• Emotional/behavioral dysregulation
• Increased risk of alcohol and/or substance use

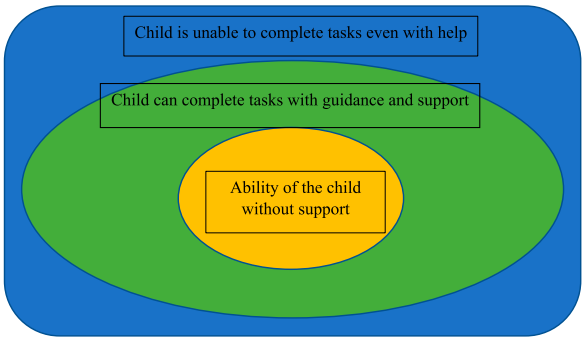


FIGURE 7
Conceptualizing the zone of proximal development.

children and adults with fetal alcohol syndrome, children with an FASD other than fetal alcohol syndrome often do not qualify for services unless they meet criteria for autism or intellectual disability despite documentation of severe impairments in adaptive function and life skills necessary to transition to adult independence.

Perhaps just as important as addressing current developmental needs of a child or adolescent with an FASD, providers should anticipate future challenges as adaptive function falls further behind age-expected abilities. Medications to target symptoms of ADHD, mood dysregulation, anxiety, depression, and sleep issues are common adjuncts to the greater implementation of environmental supports. Essential environmental supports include a calm highly structured environment with consistent routines at both home and at school. Providers often serve as advocates for services beyond school mandates for a least restrictive environment. Interventions should also address educational and vocational needs by highlighting adaptive function disabilities that often exist in the presence of borderline to normal cognitive abilities. Multiple interventions have been documented to specifically address the challenges of children with an FASD while supporting their families (88). Interventions should also address educational and vocational needs.

The transition from adolescence to adulthood is a time fraught with risks for school failure, anti-social or criminal behavior, substance use, victimization, worsening psychiatric illness, unemployment, and homelessness. Anticipation of each of these difficulties allows open discussion with parents who are frequently hesitant to discuss their concerns. Even following diagnosis of FASD, adolescents and adults with FASD remain at extreme risk for adverse outcomes. This risk is compounded by exposure to adverse childhood experiences, especially when these are frequent and enduring. Children with FASD are estimated to be at least 3.7 times more likely to have adverse childhood experiences than children without an FASD (89). Adverse experiences compound the developmental challenges of FASD to synergistically increase risk for developmental and behavioral challenges (90). Anticipatory guidance and ongoing work with families should include vigilance for adverse outcomes including school failure, recurrent school suspensions or expulsions, involvement in the juvenile justice system, sexual and interpersonal victimization, homelessness, and drug and alcohol use disorders (91,92).

FIGURE 8
Building community supports.

In addition to poor family support, adaptive difficulties in completing age-expected tasks turn simple tasks (e.g., getting to work or appointments on time, interacting appropriately with others) into insurmountable obstacles without appropriate services. Although adolescents with FASD may appear confident about managing age-appropriate tasks, this apparent self-confidence often masks impairments in adaptive skills and low self-esteem (93). Therefore, transition plans should include vocational assessment and life skills training (e.g., how to schedule and ensure appointment punctuality, following directions, appropriate workplace social behavior) as well as support in finding employment and job coaching (94). Adaptive assessments during the diagnostic evaluation process should contain information about specific weaknesses in daily living skills (e.g., hygiene, nutrition, shopping, cooking, paying bills) that are especially important during transition. Most adolescents with FASD will need ongoing support and supervision for adaptive tasks that overwhelm their capability as they attempt to meet adult responsibilities (95). Without ongoing support during transition to adulthood to accommodate deficient executive functioning and associated adaptive impairments, treatment services—no matter how extensive—are unlikely to result in a successful transition to independence and productive integration into society. Independent living programs, subsidized rent programs, and home healthcare services can facilitate a successful transition to adulthood and maximize independence (96).

Conclusion

General pediatricians and early childcare workers are often the first persons to assess a child with developmental delays. This means they are the gatekeepers for assessment and intervention long before specialists evaluate children. The lack of availability of specialists to diagnose FASD and greater lack of multidisciplinary FASD diagnostic centers, makes identification of children with an FASD in the general population an urgent public health concern. Just as interventions in other neurodevelopmental disorders improve outcomes, early identification and intervention is imperative to supporting children with an FASD. Practitioners can easily screen all children for prenatal alcohol exposure. Similarly, practitioners can begin to diagnose children with developmental delays who have a history of prenatal alcohol exposure and meet the criteria for ND-PAE. While many might argue that a diagnosis of FASD or ND-PAE is less helpful in obtaining services compared to other diagnoses such as autism, the developmental challenges and developmental trajectory of children with FASD are different from those with other neurodevelopmental disorders. Others might argue that the criteria for ND-PAE lack the sensitivity and specificity of traditional FASD diagnostic criteria. Yet traditional requirements for evaluation by a multidisciplinary diagnostic team have failed to identify the majority of children with an FASD and such centers as they currently exist will never be able to meet the demand for diagnostic and intervention services. A diagnosis of FASD offers a structure for discussion of developmental challenges with the family within the context of seeking interventions to maximize independence and prevent secondary morbidities. After 50 years of research that has increased our understanding of the effects of prenatal alcohol exposure upon neurodevelopment, front-line practitioners can use the knowledge gained from research to address the developmental and behavioral challenges of families that come to them seeking help for their children.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

DW conceptualized this project and developed an initial draft. DW and LB shared in the revisions and prepared the final version for submission.

Conflict of interest

DW previously served on the board of directors of FASD United and currently serves on the Task Force on FASD for the American Academy of Pediatrics. He receives no monetary or other recompense for these services.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Screening for fetal alcohol spectrum disorder in infants and young children

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Introduction: With an estimated prevalence of up to five percent in the general population, fetal alcohol spectrum disorders (FASD) are the most common neurodevelopmental disorder and more prevalent than autism. Early identification and subsequent early intervention have the potential to improve developmental trajectory of children with FASD. In addition, new research suggests supplementation with choline may ameliorate the developmental impairments associated with prenatal alcohol exposure. Availability of a screening tool with acceptable epidemiologic performance criteria may be clinical useful in identification of young children at increased risk for FASD. In this paper we describe the Early Fetal Alcohol Spectrum Disorder Screening Test (E-FAST) to identify young children at increased risk for an FASD.

Methods: We developed the E-FAST dataset from previously published studies, comprised of 281 children under 5 years of age, 180 (64.1%) were diagnosed with FASD and 101 (35.9%) were non-FASD.

Analysis: The analysis identified seven useful variables (prenatal alcohol exposure, ADHD (Attention Deficit Hyperactivity Disorder), foster care or adopted, small OFC (occipital frontal circumference), communication impairments, impaired social skills, and cognitive deficits. All variables were categorized as yes/no for ease of use in a screening tool. Risk ratios for each of the seven indicators were estimated using two-way table analyses. Weights for each variable were estimated based on the relative strength of their odds ratios.

Results: The average age was 2.7 years of age (S.D. 1.29) and ranged from infant (6.4%) to 4 years old (35.9%). Maternal alcohol use alone had a sensitivity of 0.97, specificity 0.65, and accuracy 0.86. For the combined seven variables, sensitivity was 0.94, specificity 0.74, and accuracy 0.87. Thus, the seven-item E-FAST screen had acceptable epidemiologic screening characteristics.

Discussion: In the United States, up to 547 infants with FASD are born each day which far exceeds the capacity of multidisciplinary diagnostic clinics. During routine clinical management of infants and young children the use of an evidence-based screening tool provides a time efficient means to exclude large numbers of young children from further follow-up for FASD. Conversely, a

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positive screen identifies a smaller number of children at increased risk for FASD requiring more intensive evaluation and follow-up.

KEYWORDS

screening, fetal alcohol spectrum disorder, prenatal alcohol exposure, neurodevelopmental disorder, infancy

Introduction

Despite widespread public information efforts to increase awareness of the risks of alcohol use during pregnancy, rates of alcohol use in pregnant women have been increasing [1, 2]. A CDC study found that 13.5% of pregnant women reported current alcohol use and 5.2% reported drinking four or more drinks on an occasion(binge-drinking) [3]. Among women of child-bearing age, 53.6% used alcohol in the past month and 18.2% binge drank [4]. Because up to 50% of pregnancies are unplanned, many of these women will have children with prenatal alcohol exposure [5]. Upon confirmation of pregnancy, most women quit or reduce alcohol use but 10.2% continue to drink [4] and recent studies indicate that over 8% of pregnant women are drinking at the end of pregnancy [6].

Prenatal alcohol exposure has been demonstrated to have a negative effect on the developmental trajectories of infants and young children language, cognitive and motor skills [7, 8]. In addition prenatal alcohol exposure increase risk for fetal alcohol spectrum disorder (FASD). FASD is the most common cause of noninheritable developmental disability in the United States. A study of first-graders in four regions of the United States found a conservatively estimated prevalence rate of 1.1%–5.0% [9]. Global prevalence of FASD is at least 2.2% with wide variation across countries and subpopulations [10]. These prevalence rates demonstrate that FASD is a disorder as common as any other medical condition physicians diagnose and treat each day. Yet both screening for prenatal alcohol exposure or for FASD is far from routine.

Even when prenatal exposure to other substances such as cannabis, cocaine, or opioids is documented in newborn medical records, screening for prenatal alcohol exposure is notably absent in most obstetric, pediatric, and child welfare records. In addition to stigma against women who use alcohol during pregnancy [11], identification of children with an FASD is complicated by the need to obtain a history of prenatal alcohol exposure. Accurate ascertainment of history of prenatal alcohol exposure is complicated by the frequency in which children at the highest risk for an FASD often do not reside with their biological parents. In a recent study of 151 children/young adults screened for FASD, only 4 (2.6%) were raised by biological family members. Information on prenatal care and alcohol and other substance exposure can be very challenging to obtain in these circumstances [12].

Another challenge in the diagnosis of FASD is the lack of a screening tool for practitioners to utilize to easily identify infants and young children at risk for an FASD. A comprehensive review of FASD screening tools identified 20 unique screening tools for FASD utilized in 45 cross-sectional or case-controlled studies [13]. Typical screening tools analyze facial dysmorphology, growth retardation, behavioral and developmental indicators, along with characteristics of parents. Of the 20 screening tools, only 5 studies included children under the age of six and only 3 included children ages 2 to 3; no studies included children under 2. Another recent study retrospectively analyzed 151 subjects all of which were seen in a national FASD clinic [12]. The ages in this study ranged from 3.75 to 22 years. Within this range, 78% (118 of 151) were between ages 6 and 16 and no data was presented for children under age 3.7 years. These two articles demonstrate the need for an early screening for FASD to allow early identification and intervention to maximize child neurodevelopment. In this manuscript we discuss a new screening tool for FASD which was developed for use in young children during routine healthcare visits or when developmental delays are a concern.

In this manuscript we respond to this issue by reporting on the development of a screening test for FASD for very young children (Early Fetal Alcohol Spectrum Disorder Screen for Young Children) the E-FAST.

Methods

The E-FAST data set was developed from three deidentified data sets which have been previously published (FAS Diagnostic Checklist 2002; $n = 405$, FAS Screen 1997; $n = 264$, and data from the ARND (alcohol related neurodevelopmental disorder) Behavioral Checklist $n = 47$. The initial criteria for diagnosis is determination of prenatal alcohol exposure. The diagnostic criteria and the methodology for diagnosis has been presented in detail in [14]. In brief, each child with a diagnosis of FASD had an exposure assessment which consisted of the One-Question Screen "When was your last drink?", and a Maternal Risk Score and a dosimetry assessment [14–16]. This data is then reviewed and a five item Likert scale is used to assess clinician confidence in exposure. The five scale intervals are confirmed prenatal alcohol exposure, prenatal alcohol exposure, no-reporter, no exposure and confirmed no-prenatal alcohol exposure. The dosimetry assessment collects data on drinking days per week, drinks per drinking day, number of binge episodes

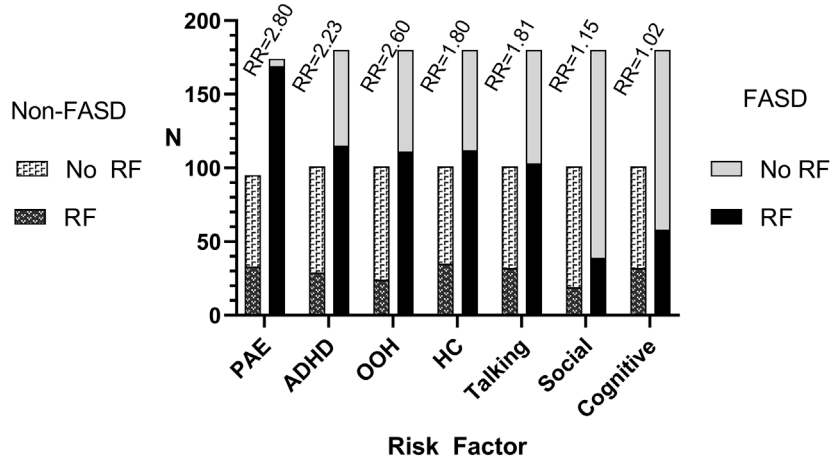

FIGURE 1
The number of young children with fetal alcohol spectrum disorder (FASD) and non-FASD comparison children who had or did not have one of the seven E-FAST risk factors included in the analysis. PAE, prenatal alcohol exposure; ADHD, attention deficit hyperactivity disorder; OOH, foster care, adopted, living out of parents home; HC, head circumference; Talking, verbal communication impairments; Social, social skill deficits; Cognitive, cognitive impairments.

TABLE 1 Logistic regressions for different combinations of variables for the E-FAST Screen for FASD and the assigned variable weights for the items included in the screen.

N	E-FAST variables	E-FAST variable #	B Est	*P*	OR	Weight
	Model 1					
269	PAE	1	2.176	<.001	63.502	20
	Model 2					
234	ADHD	2	2.5081	<.001	12.282	10
	OOH	3	1.0584	.0044	2.882	3
	HC	4	1.5070	<.001	4.513	5
	Talking	5	0.1725	.6629	1.188	1
	Social	6	0.2975	.7309	1.346	1
	Cognitive	7	−0.0102	.9837	0.990	1
	Model 3					
222	PAE	1	3.2671	<.001	26.236	20
	ADHD	2	2.4848	<.001	11.999	10
	OOH	3	0.9122	.0497	2.490	3
	HC	4	1.3010	.004	3.676	5
	Talking	5	0.1173	.8154	1.124	1
	Cognitive	7	0.1990	.7672	1.220	1

PAE, prenatal alcohol exposure; ADHD, attention deficit hyperactivity disorder; OOH, foster care, adopted, living out of parents home; HC, head circumference; Talking, verbal communication impairments; Social, social skill deficits; Cognitive, cognitive impairments.

(four or more standard drinks on an occasion), what is a drink(s) and days per week for cigarette smoking and number of cigarettes per smoking day. The second criterion includes meeting the neurobehavioral phenotype for FASD [14, 17]. This included assessment of relevant records for previous intellectual testing, neuropsychological testing, adaptive behavior testing, assessment for attention deficit hyperactivity disorder, speech and language testing, memory testing, executive function testing, vision and

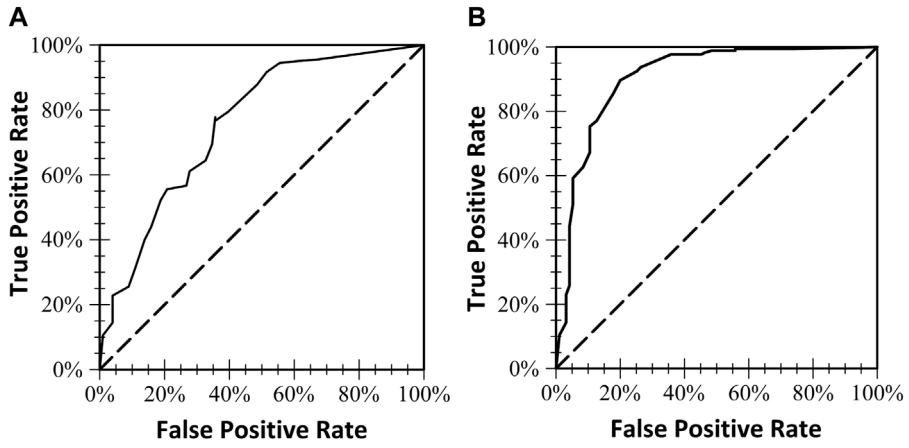

FIGURE 2
The two receiver operating characteristics (ROC) curves and the area under the curve for each of the two models. **(A)** Included six of the seven E-FAST Screening variables (prenatal alcohol use was not included in this model). **(B)** Models all seven E-FAST screening variables.

TABLE 2 Receiver operating characteristics (ROC) analysis using suggested cutoffs for combinations of screening variables for FASD three variable models of the E-FAST. The E-FAST variables numbers are in parenthesis.

N	Cutoff	Variables	Sensitivity	Specificity	Accuracy	AUC
269	≥20	PAE (1)	.9713	.6526	.8587	.8119
281	≥7	ADHD (2), OOH (3), HC (4), Talking (5), Social (6), and Cognitive (7)	.7778	.6436	.7295	.7579
269	≥21	PAE (1), ADHD (2), OOH (3), HC (4), Talking (5), Social (6), and Cognitive (7)	.9368	.7368	.8662	.9061

PAE, prenatal alcohol exposure; ADHD, attention deficit hyperactivity disorder; OOH, foster care, adopted, living out of parents home; HC, head circumference; Talking, verbal communication impairments; Social, social skill deficits; Cognitive, cognitive impairments.

hearing testing, number of adverse childhood experiences, and number of foster home placements. Where needed additional testing was completed during the assessment or by referral. Children with other comorbid disorders were not excluded from the FASD group if they met criteria for FASD.

The ARND data set ($n = 47$) did not include data on occipital frontal head circumference (OFC) or the size of the child (height or weight). Those values were imputed using estimated values from a logistic regression that used smoking, age, sex, and race as input variables.

Determination of screening variables

The final dataset included 281 children under age 5 years included in the analysis. All variables were categorized as yes/no for ease of use as variables in a screening tool. Occipital frontal circumference (OFCs) under the 20th percentile for their age were considered at risk. Children placed in foster care or adopted were combined into one variable. The variable ADHD included a diagnosis of ADHD and behavioral observations of attention deficits or impulsiveness. Speech and language disorders included diagnosed speech and language disorders, stuttering, or observations of communication impairments. The variable social problems included social skills deficits, difficulty or inability to make friends, or noticeable deficits in relating to other children. Cognitive impairments included IQ below 85, learning disability, memory impairments, need for special education services, therapy, or early intervention services due to learning deficits.

Multiple other variables were available but not included in the analysis. They included maternal variables such as age and smoking status, information on the father or siblings (substance use, diagnoses, mortality), and variables regarding the child such as other diagnosis or birth information. These were excluded since the variables did not have sufficient observations or positive values to be useable. Other variables such as mother's age, were so closely related to other variables their collinearity rendered them unusable. Birthweight, which had to be controlled for gestation, was too complicated and OFC gave an easier and quick measure of a child's size. There were no facial or other

physical features included other than OFC. Other variables like depression were not included since these are typically diagnosed in older children.

Statistical analysis

The initial analysis of the E-FAST dataset was designed to identify useful screening variables. Risk ratios for each of the potential indicators were estimated using two-way table analyses. Multiple logistic regressions of the variables predicting FASD were estimated for just maternal drinking alone, the other six variables, and all seven variables together. From these regressions, weights for each variable were estimated based on the relative strength of their odds ratios. Composite variables from different combinations of indicator weights were summed and used to produce receiver operating characteristic curves (ROC). Variable combinations included 1) maternal alcohol exposure alone, 2) ADHD, speech and language disorders, social skill deficits, cognitive impairments. These in turn were used to estimate cutoff values for the composite variable models that maximized sensitivity and specificity of the indicators.

Results

Of the 281 children who had screening variables useable for this analysis, 180 (64.1%) were diagnosed with FASD and 101 (35.9%) were non-FASD. In this sample 161 (57.5%) were male and 84 (29.9%) were white. The average age was 2.67 years (S.D. 1.29) and ranged from infant (6.4%) to 4 years old (35.9%).

The initial analysis of the E-FAST dataset identified seven variables that were useful. Figure 1 presents the number of children with any of the seven screening variables by FASD group (FASD or non-FASD). Alcohol use during pregnancy (variable 1) was reported in nearly all children with a diagnosis of FASD. Children diagnosed with FASD were over twice as likely to have ADHD (variable 2) or be in foster care or adopted (variable 3), and nearly twice as likely to have small OFC (variable 4) or communication impairments (variable 5). Children with FASD were also at increased risk for socialization (variable 6) or cognitive deficits (variable 7). Table 1 shows the logistic regressions of different combinations of E-FAST risk factors. Alcohol use during pregnancy had the highest odds ratio of 63.5. When the six risk factors were taken together, without alcohol use, ADHD had the highest OR of 12.3, Children who were in foster care or adopted OR was 2.9, and small OFC OR was 4.5. Talking, social, and cognitive difficulties were not significant with ORs near one. Weights for composite scores, based on ORs, ranged from 20 points for alcohol to 1 point for cognitive impairments. Figure 2.

ROC curves were used to find cutoff scores for the screening combinations of the weights. Table 2 shows the sensitivity, specificity, accuracy, and area under the ROC curve (AUC) for maternal alcohol use alone, risk factors without alcohol use, and all variables together. Sensitivity of the E-FAST was highest for the two models where alcohol use during pregnancy data was available (.97 and .94), though specificity increased with the addition of the other six risk variables (.74). Although the E-FAST ROC values were lowest for positive screening scores without alcohol use, the six variable model accuracy was still over 70%.

Figure 3 is the final E-FAST Screening tool. Scoring the E-FASD is simple. If the score exceeds 7, the screen is positive and this suggests the child is at increased risk for having and FASD. Among the options for the clinician is placing the child in a more intensive follow-up system to increase the frequency of well child visits. This effort could include increased screening for common problems these would include vision and hearing, sleep, speech and language delays. Identification of exposure to adverse experiences of childhood or placement in foster care would suggest increased risk for FASD. In the accompanying manuscript we describe an office-based approach for identification and management FASD developed for pediatricians and other pediatric providers (8).

Discussion

In the United States, FASD prevalence rates are as high as one in 20 school-aged children or about 5% of first grade students (7)This suggests that in an annual birth cohort of 4.0 million births in the United States approximately 200,000 are infants are born with FASD each year. This figure equates to 3,800 infants with FASD born each week or 547 every day. Only a very small fraction of children with FASD can be seen in the few multidisciplinary clinics currently operating in the United States. Strategies for office-based identification by pediatricians and other early child health providers is urgently needed to facilitate identification and intervention in early childhood [8]. One potentially useful strategy is the E-FAST which can be used to exclude a majority of infants and young children from those who require further assessment for FASD. This is an important function of screening in an office-based practice. The E-FAST functions has acceptable epidemiologic performance characteristics and clinicians could expect that most infants and young children with a negative E-FAST screen will not have FASD.

The second role of screening is to identify a population of infants and young children requiring further assessment. The E-FAST is useful for screening for potential cases since some of the common features of FASD are included as variables in the screen. Lastly, a history of prenatal alcohol exposure may often be a key in the differential diagnosis of FASD. On the E-FAST a positive finding of maternal alcohol use represents a positive screen. However, where information on prenatal alcohol exposure is not available the other

EARLY FETAL ALCOHOL SPECTRUM DISORDER SCREENING TEST FOR INFANTS AND YOUNG CHILDREN (E-FAST)

for use with infants and young children - birth to age three years

Name_____ Date_____

DOB_____ Age_____ Sex M F

		Circle positive item
1.	Any alcohol use during pregnancy-Prenatal alcohol exposure	20
2.	Signs or concerns about attention deficit hyperactivity disorder (ADHD)	10
3.	Out-of-home placement (foster care, adoption, lives with relatives)	2
4.	Small head size (occipital frontal circumference OFC) below 20th percentile	4
5.	Verbal communication delays	1
6.	Poor socialization, difficulty interacting with others, few friends	1
7.	Cognitive impairment (delays in learning)	1

Screen is positive if score is 7 or more **Total Score**

FIGURE 3
The early fetal alcohol spectrum disorder screening test for infants and young children (E-FAST).

six variables can be used to screen. Since information on prenatal alcohol exposure may not always be accurate, the other six variables on the E-FAST can provide a rational for ongoing observation and alternative strategies for exposure assessment.

Concerns about ADHD prior to diagnosis would also provide a useful rational for ongoing monitoring for FASD as well since 50% of children diagnosed with FASD also have either ADHD or concerns about ADHD [18]. FASD is much more prevalent among children in foster care or who have been adopted [19]. The E-FAST can be used in this setting where maternal disclosure of alcohol use has potential for hindering reunification or when direct interview of the mother is not possible.

The primary gatekeepers to identification of children with an FASD are general pediatricians and other front-line early childhood providers. Children identified by the E-FASD screen as at risk for FASD, can be readily referred for further evaluation. Given the paucity of FASD diagnostic centers in the United States especially in rural areas, further assessment might be completed by telemedicine evaluation which has the potential to dramatically increase the number of children diagnosed with an FASD [20]. In the management of children in foster care or with developmental disorders routine screening for exposure to prenatal alcohol is an important step. The basis of FASD diagnosis rests upon prenatal alcohol exposure. The E-FAST-screening tool should not be considered an alternative to screening all children for prenatal alcohol exposure which is a separate clinical issue. The E-FAST offer a rapid means for front-line practitioners to screen, identify, and refer children for FASD diagnosis while also providing a rational for starting intervention services. Finally, new research suggests that pre- and even post-natal choline supplementation may have potential for mitigating the effects of prenatal alcohol exposure [21]. While evidence for the benefits of choline supplementation remains sparse, many experts and families may decide to use choline given the minimal risk of side effects of supplementation in children at risk for FASD.

This study is limited by lack its relatively small sample size, limited inclusion of children from a wide range of diverse settings, cultures and ethnic diversity. When other tools become available for use in this young population comparative studies should promptly be initiated to contrast different approaches to determine optimal screening strategies. It may be that different diagnostic criteria for FASD would result in different screening variables and these variables may have different screening weights in other screening tools.

Further areas of study should also include population-based application of the E-FAST in general pediatric settings to assess the efficiency, efficacy and effectiveness of the tool. Such studies may also refine the performance characteristics of the tool. In addition, further pathways for referral of children screening positive should be clarified to help practitioners know about next steps after a positive screen or diagnosis of FASD [8]). Despite these limitations, this study provides an initial strategy to improve the identification of children with FASD. More importantly, the E-FAST screen has the potential for use to address under identification of children with FASD. Early identification and entry into services is an important management strategy for children and families impacted by FASD. This screening tool may be a part of a system to identify young children who are currently undiagnosed and a first step in entry into appropriate intervention services.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by University of North Dakota Institutional Review Board. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

LB, MK, LF, and CS conceptualized the study. MK completed the analysis and contributed to the results. LB, DW, LF, and CS developed the results and discussion sections. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Methadone alters the peripheral inflammatory and central immune landscape following prenatal exposure in rats

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Opioid use during pregnancy continues to rise at alarming rates with a parallel trend in the number of infants and children exposed to opioid medications each year. Prenatal opioid exposure (POE) occurs at a critical timepoint in neurodevelopment disrupting intricate pathways essential for neural-immune maturation with the potential for devastating long-term consequences. Understanding the mechanisms underlying injury associated with POE is essential to address long-term outcomes and identify diagnostic and therapeutic biomarkers in this vulnerable patient population. Using an established preclinical model of POE, we investigated changes in cerebral and peripheral inflammation and peripheral blood mononuclear cell (PBMC) activity. We hypothesized that neuroinflammation, as defined by changes in specific cerebral immune cell populations, would exist in adult rats following POE concomitant with sustained peripheral immune hyperreactivity (SPIHR). Our data demonstrated alterations in cerebral immune cells at postnatal day 60 (P60) typified by increased regulatory T cells ($p < 0.01$) and neutrophils ($p < 0.05$) in rats with POE compared to controls. Evaluation of serum revealed increased levels of IL-6 ($p < 0.05$) and CXCL1 ($p < 0.05$) at P21 in rats with POE compared to controls with no significant difference in cytokine or chemokine levels between the two groups at P60. Additionally, PBMCs isolated from rats with POE at P21 demonstrated baseline hypersecretion of IL-6 ($p < 0.01$) and SPIHR with increased levels of TNF-α ($p < 0.05$) and CXCL1 ($p < 0.05$) following stimulation with LPS. At P60, however, there was no significant difference found in cytokine or chemokine levels secreted by PBMCs isolated from rats with POE at baseline or with LPS stimulation when compared to controls. Taken together, these data demonstrate cerebral inflammation months after prenatal opioid exposure and long after the resolution of systemic inflammation and SPIHR seen at toddler

age equivalent. Chronic alterations in the cerebral immune cell populations secondary to prenatal opioid exposure may underly long-term consequences of developmental brain injury including deficits in cognition and attention. These findings may be invaluable to further investigations of precise biomarkers of injury and targeted therapeutics for this vulnerable population.

KEYWORDS

methadone, inflammation, prenatal opioid exposure, immune priming, PBMC, SPIHR

Introduction

The crisis of opioid use in the United States continues to grow and has a significant impact on many populations including pregnant women and children. Opioid use disorder during pregnancy has risen at alarming rates in the past decade with a 131% increase from 2010 to 2017 in women with a maternal opioid-related diagnosis at time of delivery (1). Paralleling this epidemic has been a sharp increase in the number of infants exposed *in utero* to opioid medications each year. Neonatal opioid withdrawal syndrome (NOWS) is a well-recognized consequence of prenatal opioid exposure (POE) in the first few weeks of life leading to an extended length of hospitalization for many newborn infants. However, the long-term adverse outcomes associated with POE are just beginning to be understood (2). As the epidemic grows, understanding the mechanisms underlying POE and the long-term consequence of this exposure is paramount to supporting the health and development of this vulnerable population.

Prenatal opioid exposure occurs as a result of maternal use or misuse of prescription opioid medications including oxycodone, hydrocodone, morphine, codeine and fentanyl, or illicit opioids, such as heroin. While it is known that POE is associated with an increased risk of fetal growth restriction and preterm birth, the effects of POE on the developing central nervous system remain poorly understood (3–5). Clinical data shows evidence of abnormal brain development with decreased brain volumes and aberrant structural connectivity in children exposed to opioid medications prenatally (6–12). Others have shown significant cognitive and motor dysfunction in school-age children with POE when compared to age-matched peers without prenatal opioid exposure (13). Larger cohort studies are limited by social, economic, and environmental confounding factors, but demonstrate higher risk of attention deficit hyperactivity disorder and symptoms in school-age children with prenatal opioid exposure as well (3, 14). Elegant preclinical studies using animals show similar findings and elicit concern for long-term neurobehavioral consequences in this patient population. Specifically, prenatal methadone exposure changes open field activity, and impairs sensorimotor developmental milestone acquisition concomitant with reduced neuronal density in motor cortex and aberrant circuit connectivity (15). Prenatal Methadone and buprenorphine cause impaired recognition memory, and nonspatial reference learning in young adult rats (16) corroborating reports of impaired cognitive flexibility and learning acquisition older adults after POE using a touchscreen platform (17). Similarly, buprenorphine and methadone impair social interaction and novel object recognition, diminish elevated plus maze performance, and induce anxiety (18). Studies using other opioids yield similar results. Prenatal fentanyl exposure alters sensory processing defined by multiple changes in synapses, regional changes in excitatory and inhibitory tone, and diminished dendritic arbor (19). Prescription opioids, including oxycodone, disrupt afferent regulation of dopamine activity in the ventral tegmental area during development suggesting disruption of the trajectory of mesolimbic circuity maturation and providing connection to common neuropsychological outcomes such as anxiety, attention, and depression (20).

Understanding the mechanism of opioid-induced neural injury and the lasting impact of this injury across the lifespan is key to identifying therapeutic targets and tailoring intervention to high-risk patients at crucial times in development. The ability to recognize patients at higher risk of long-term adverse outcomes from POE with precise biomarkers is also essential in guiding clinical management for diagnosis and treatment (21–23). Here, we defined alterations in the immune system following POE to test whether adults with POE had persistent brain inflammation. Specifically, we conducted investigations of serum inflammatory cytokine and chemokine profiles and evaluated peripheral immune hyper-reactivity from toddler age equivalent at P21 to an adult human age equivalent at P60. We also used multiparameter flow cytometry to interrogate cerebral and peripheral blood immune cell population dynamics in adulthood. We hypothesized that POE would induce neuroinflammation that could be detected in adulthood, defined by changes in infiltrating immune cells. We also predicted there would be sustained inflammation alongside disruption in the function and composition of the peripheral immune system.

Methods

Animals

Sprague-Dawley rat dams and litters were maintained in a temperature and humidity-controlled facility with food and

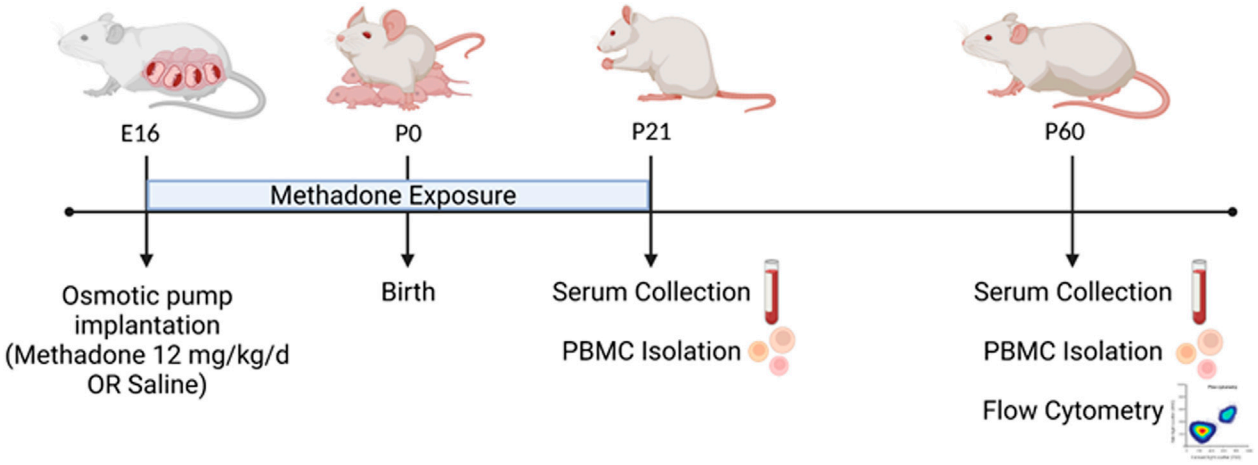

FIGURE 1
POE and Experimental Paradigm. Osmotic mini pumps were implanted into pregnant rat dams at embryonic day 16 (E16) for continuous methadone or saline exposure. Rat pups were born at term on E23/postnatal day 0 (P0) and continued to receive methadone or saline through maternal milk until weaning at P21. At P21 and P60, serum was collected and PBMCs isolated from all animals. At P60, brain and PBMCs were also collected for multiparameter flow cytometric analyses (Figure created with Biorender.com).

water available *ad libitum*. A 12-h dark/light cycle was maintained for all animals with lights on at 0800 h. All experiments were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Johns Hopkins University School of Medicine. Protocols were developed and performed consistent with National Research Council and ARRIVE guidelines (24). Litter size was similar between methadone-exposed and saline-exposed litters. As previously published, (17) weights were significantly lower in methadone-exposed litters as compared to saline-exposed litters. For each experiment described, the data represents true n (individual rats). For every experiment and outcome measure, we used offspring from at least 4 different litters per condition to control for litter effects. Male and female offspring were used in every outcome measure, and in approximately equal numbers where possible and dictated by experimental endpoint.

Methadone exposure

Per previously published methods, on embryonic day 16 (E16) osmotic mini pumps (ALZET, Cupertino, California) were implanted subcutaneously in the nape of the neck of pregnant rat dams for 28 days of continuous methadone (12 mg/kg, 0.25 µL/h flow rate) or sterile saline infusion (Figure 1) (17, 25). Methadone is a synthetic, long-acting, µ-opioid receptor agonist that readily crosses the placenta and blood-brain barrier. Specifically, following induction and maintenance of anesthesia with inhaled isoflurane, dams underwent minipump placement with a 1.5 cm transverse skin incision followed by careful blunt dissection of the subcutaneous space. Osmotic pumps were prefilled and primed prior to insertion, followed by closure of the space with sutures. The total duration of anesthesia was no longer than 7 min. Dams were carefully monitored following the procedure for full recovery. Rat pups were born at E23/postnatal day 0 (P0) following completion of gestation and remained with their dams. Pups continued to receive methadone or saline through the maternal milk supply until weaning on P21 (17, 25). Daily health checks were performed for pup wellbeing.

Blood and brain collection

At P21 and P60, brain and blood were collected. Specifically, at the conclusion of each experiment, rats were deeply anesthetized, and venous blood was collected from the right atrium in pyrogen-free, K2 EDTA, vacutainers (BD Vacutainer, Franklin Lakes, NJ). Whole blood was then aliquoted as dictated by endpoint assays to further undergo PBMC isolation or serum separation. Brains from each animal were harvested at the time of blood collection.

Flow cytometry

At P60, brain and peripheral blood mononuclear cells (PBMCs) were collected from adult rats for flow cytometry consistent previous reports (17, 25–31). Using a Miltenyi adult brain dissociation kit and Miltenyi gentleMACS™ protocol,

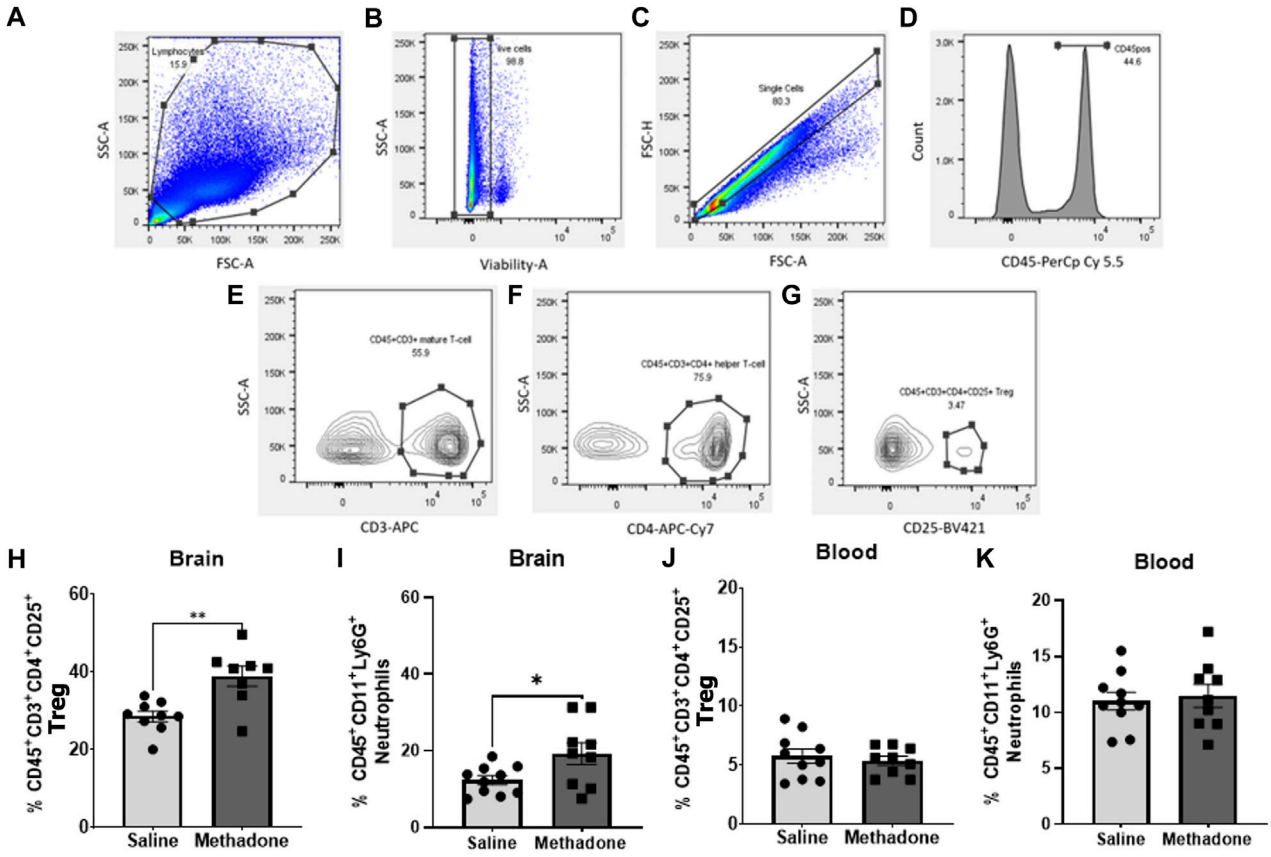

FIGURE 2
Adult rats have significantly altered central immune cell population dynamics following POE. Multiparameter flow cytometric analyses were used to identify changes in lymphocyte populations in blood and brain isolated from rats with POE and controls at P60. Lymphocytes were identified by size, granularity, and viability dye staining **(A–C)**. Further gating for CD45 **(D)**, CD3 **(E)**, CD4 **(F)** and CD25 **(G)** is shown. Increased regulatory T cells **(H)** and neutrophils **(I)** were observed in the brains of rats with POE compared to controls. No significant differences were found in regulatory T cells and neutrophils **(J,K)** in PBMCs isolated from rats with POE and controls (Mann-Whitney U-test for all, $*p < 0.05$, $**p < 0.01$, FSC, forward scatter; SSC, side scatter).

whole cerebrum was digested for single-cell suspension (22–24). This suspension was passed through 70-µm cell filters and then underwent debris elimination using debris removal solution. Miltenyi red blood cell lysis solution (1x) was then used for complete removal of erythrocyte populations. Live cells were counted on a Countess™ II Automated Cell Counter (Thermo Fisher Scientific). Next, $1x10^6$ live cells were incubated with a saturating solution of Fc block (Clone D34-485, BD Biosciences, San Jose, CA) followed by staining with fluorochrome-conjugated viability dye and antibodies against: CD45-PerCp Cy5.5 or CD45-APC Cy7 (Clone OX1; eBiosciences, Waltham, MA), CD11b/c BV605 (Clone OX42; eBiosciences, Waltham, MD), Ly6G-FITC (Clone RB6-8C5, AbCam, Cambridge, MA), CD3-APC (Clone 1F4; BD Biosciences, San Jose, CA), CD4-APC Cy7 (W3/25; Biolegend, San Diego, CA), and CD25-BV421 (OX-39; BD Biosciences, San Jose, CA). Data were acquired using a BD LSR-II flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software v.10.7.1 (FlowJo LLC, Ashland, OR). Cells were first gated based on size and granularity (forward scatter (FSC) vs. side scatter (SSC)), followed by gating on live cells confirmed with viability dye staining. Single cells were then identified using FSC-A vs FSC-H. CD-45$^+$ cells were identified and further analyzed for CD 11b/c expression. CD45$^+$CD11b/c$^+$ cells could then be gated for Ly6G expression to identify neutrophils (27, 32, 33). In the brain, CD45$^+$ cells were further characterized. CD45^{high}CD11b/c$^+$ cells were considered macrophages and CD45$^{low/med}$CD11b/c$^+$ cells were considered resident microglia (23, 27, 28). Using a separate panel, T cells were identified by assessing CD45$^+$CD3$^+$ cells. CD45$^+$CD3$^+$CD4$^+$ were classified as helper T cells and CD45$^+$CD3$^+$CD4$^+$CD25$^+$ as regulatory T cells (Tregs) (22, 23, 29, 30) (Figures 2A–G).

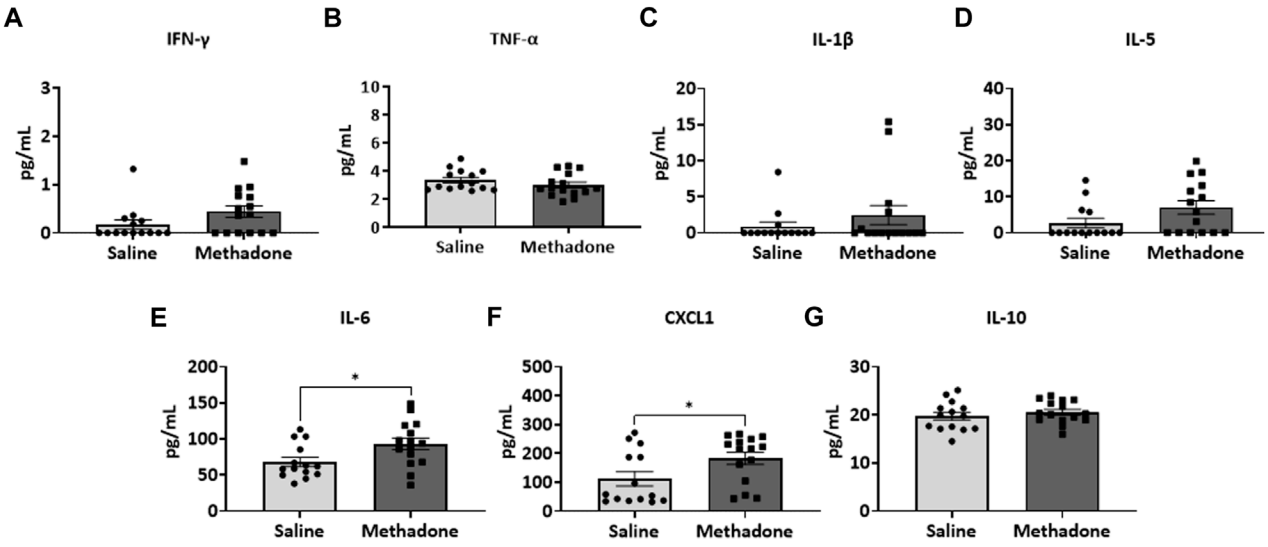

FIGURE 3
POE leads to elevations in serum inflammatory cytokines and chemokines at P21. Osmotic mini pumps with methadone or saline were implanted in pregnant dams at E16. Pups were born and serum was collected at P21 and assayed using a translatable multiplex electrochemiluminescent biomarker platform. At P21, methadone-exposed rats demonstrate significantly elevated levels of IL-6 **(E)** and CXCL1 **(F)** compared to controls reflecting persistent peripheral inflammation following POE at a toddler age equivalent. (t-test for all, $*p < 0.05$).

Serum collection

Consistent with published methods, whole blood at P21 AND P60 was centrifuged at 6000 x g for 15 min at 4°C (17, 27, 32–36). Serum was then removed and stored at −80°C until cytokine and chemokine analysis. Repeated freeze-thaw cycles were avoided.

Peripheral blood mononuclear cell isolation

PBMCs were isolated from saline-exposed and methadone-exposed rats using a Ficoll-gradient separation consistent with previously published methods (17, 25–27). In sterile 15-ml conical tubes, equal volumes of venous blood and RPMI (Roswell Park Media Institute) 1640 media (Gibco, Waltham, MA, United States) were placed in a layer on top of Ficoll-Plaque Plus (GE Healthcare, Chicago, IL, United States) and centrifuged at 400 x g for 30 min at room temperature. The PBMC cell layer was then collected and transferred into a new 15 ml conical tube and resuspended in RPMI media. Two wash cycles with RPMI media were performed by centrifuging the sample at 400 x g for 10 min at room temperature, followed by supernatant disposal and resuspension of the pellet. Following the wash cycles, the PBMC cell pellet was resuspended in media and a cell density of 1×10^6 cells/mL per well was plated in duplicate on 3.5 cm Petri dishes.

Peripheral blood mononuclear cell treatment with LPS

PBMCs from saline-exposed and methadone-exposed rats were plated and treated with media only or stimulated with LPS at a concentration of 100 ng/ml (17, 25–27). Media and cells were collected at 3 h after stimulation and 24 h after stimulation to assess PBMC secretory activity and changes prior to and after protein synthesis. Cells and supernatant were stored in sterile tubes at −80°C until further analysis. Each culture, condition and exposure were performed in duplicate. Repeat freeze-thaw cycles were avoided.

Multiplex electrochemiluminescent immunoassay

Cytokines and chemokines in serum samples and supernatant from cultured PBMCs (secretome) were analyzed using a V-PLEX Proinflammatory Panel 2 Rat Kit (K15059D; Meso Scale Diagnostics, Rockville, MD, United States) (17, 18, 22, 23, 25, 26, 31, 32). The following cytokine and chemokine secretions were assessed: interferon gamma (IFN-γ), interleukin-1β (IL-1 β), IL-4, IL-5, IL-6, IL-10, IL-13, chemokine (C-X-C motif) ligand 1 (CXCL1) and tumor necrosis factor-α (TNF-α). The assay was performed according to manufacturer specifications. Each sample of PBMC culture media and serum was diluted 1:3 and loaded in duplicate with prepared standards onto blocked and washed 96-well plates. Following a series

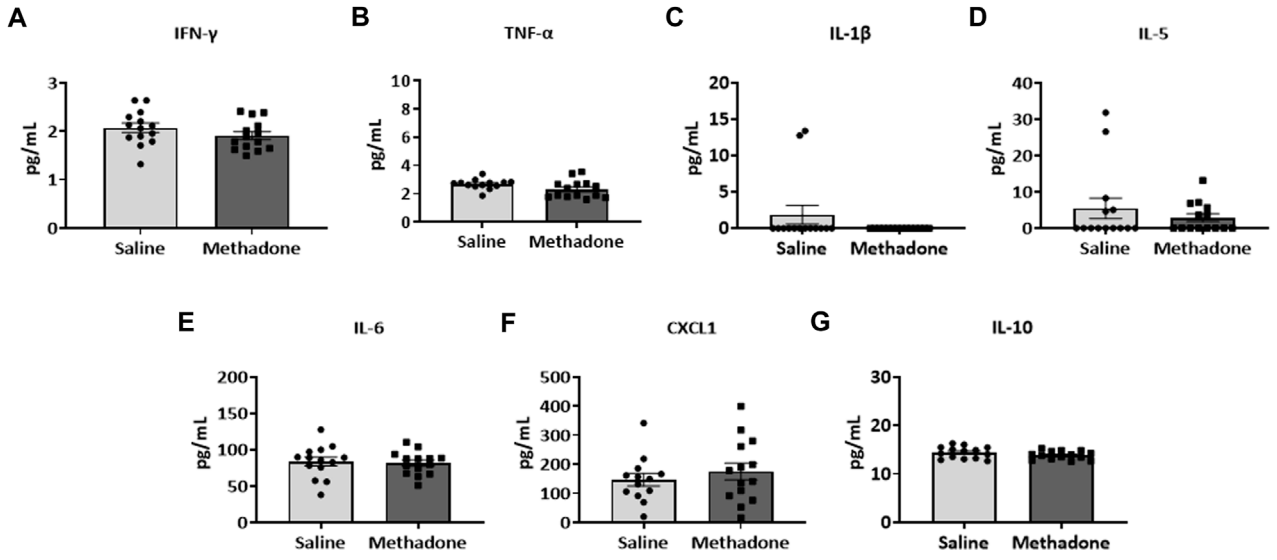

FIGURE 4
Peripheral inflammation secondary to prenatal opioid exposure normalizes by P60. Osmotic minipumps primed with methadone or saline were implanted in pregnant dams at E16. Pups were born and serum was collected at P60 and assayed using a translatable multiplex electrochemiluminescent biomarker platform. At P60, no significant differences were observed between methadone-exposed and saline-exposed rats (t-test for all).

of washes and incubation with antibody detection solution, plates were washed and loaded with read buffer onto a Quickplex SQ 120 Imager (25–27, 37–40). Consistent with the standard in the field, samples reading below the detectable limit of the assay or with a coefficient of variation greater than 25% in an individual assay were removed from further analysis (25–27, 37–40). The V-PLEX pro-inflammatory panel assay is performed with less than 10% variability between runs and sensitivity in sub pg/mL ranges.

Statistical analysis

Data are represented as mean ± the standard error of the mean (SEM). Data was tested for normality using the Shapiro-Wilk test. Statistical differences between 2 groups of parametric data were established with Student's *t*-test and non-parametric data with the Mann-Whitney test with $p < 0.05$ considered statistically significant. GraphPad Prism 9.3.1 software was used to perform statistical analyses.

Results

Prenatal opioid exposure induces changes in central immune cell populations

At P60, using multiparameter flow cytometric analyses, PBMCs and brain immune cell populations were examined in-depth for rats with POE versus controls (n = 10/group (5 males; 5 females) for saline, n = 9/group (5 males; 4 females) for methadone). Flow cytometric analyses of the brain revealed immune cell population changes following POE, with a significant increase in regulatory T cell ($CD45^+CD3^+CD4^+CD25^+$) populations in rats with POE as compared to controls (saline: 28.57 ± 1.35%, methadone: 38.95 ± 2.58%, mann whitney U-test, $p < 0.01$) (Figure 2H). Furthermore, there was a significant increase in $CD45^+CD11b/c^+Ly6G^+$ neutrophils in the brains of rats with POE as compared to controls (saline: 12.33 ± 1.18%, methadone 19.24 ± 2.86%, mann whitney U-test, $p < 0.05$) (Figure 2I). There was no significant difference in helper T cell ($CD45^+CD3^+CD4^+$) populations, resident microglia ($CD45^{low/med}CD11b/c^+$) or infiltrating macrophages ($CD45^{high}CD11b/c^+$) in the brain (data not shown). Flow cytometric analyses of immune cells in the blood revealed no significant differences in helper T cells, regulatory T cells, infiltrating macrophages, or neutrophils (Figures 2J,K). Overall, these data demonstrated cerebral inflammation at P60 defined by increased regulatory T cells and neutrophils in rats with POE.

Peripheral inflammation from POE is evident beyond the neonatal period

Detailed investigation of inflammatory markers in serum was undertaken at both P21 and P60 to establish potential

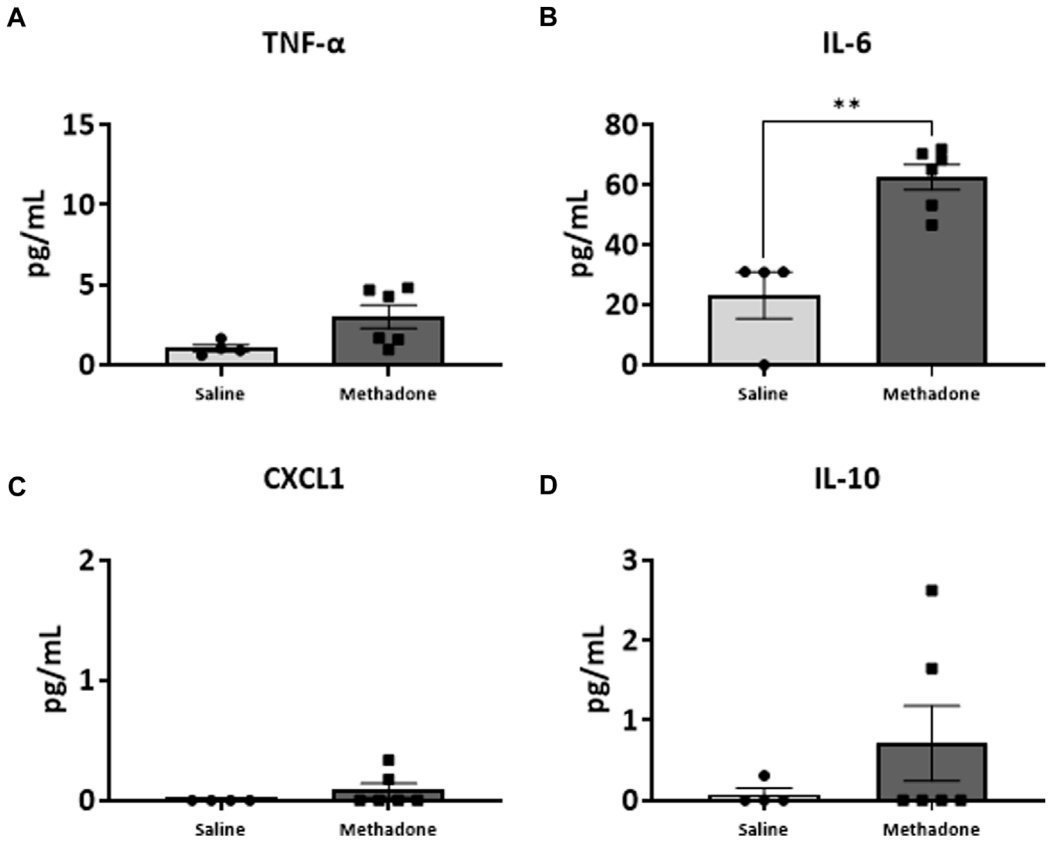

FIGURE 5
POE causes alterations in the PBMC secretome at baseline. PBMCs were isolated from P21 pups prenatally exposed to saline or methadone. Conditioned media was then assessed for cytokines and chemokines after 3 h in culture. PBMC secretome from rats with POE demonstrated significant elevation in IL-6 compared to controls. There was no significant difference in other cytokine and chemokine levels between the two groups (t-test for all, $^{**}p < 0.01$).

differences in secreted proteins with methadone exposure and inflammatory network activation. Analysis of the serum following POE began with measurement of cytokine and chemokine levels at P21, approximately human toddler age equivalent (n = 15/group; 7 males; 8 females) (33). Rats with POE had a significant elevation in IL-6 (saline: 68.11 ± 6.34 pg/ml, methadone: 93.08 ± 8.11 pg/ml, t-test, $p < 0.05$) (Figure 3E) and CXCL1 (saline: 111.9 ± 24.8 pg/ml, methadone: 183.6 ± 21.2 pg/ml, t-test, $p < 0.05$) compared to controls (Figure 3F). At P60, an adult equivalent age (23), evaluation of serum cytokine and chemokines revealed no statistically significant difference in levels of IFN-γ, TNF-α, IL-1β, IL-5, IL-6, CXCL1, and IL-10 when comparing rats with POE to controls (n = 14/group; 7 males and 7 females) (Figure 4). In summary, this data shows increases in pro-inflammatory serum biomarkers at P21 following POE, defined by significant elevation of IL-6 and CXCL1 in rats with POE, and normalization of serum inflammatory markers at P60.

POE alters the baseline PBMC secretome

Following our assessment of peripheral serum cytokine and chemokine levels, we further investigated peripheral immune system reactivity with evaluation of the baseline and stimulated PBMCs secretome at P21 (n = 4/group for saline; 2 males; 2 females, n = 6/group; 3 males; 3 females for methadone). At baseline, the conditioned media of PBMCs isolated from rats with POE demonstrated elevated levels of TNF-α levels at baseline in rats with POE after 3 h in culture (saline: 1.051 ± 0.220 pg/ml, methadone: 3.009 ± 0.721 pg/ml, t-test, $p = 0.0655$) although this failed to reach statistical significance (Figure 5A). IL-6 was significantly elevated at baseline compared to control rats (saline: 23.22 ± 7.74 pg/ml, methadone: 62.56 ± 4.19 pg/ml, t-test, $p < 0.01$) (Figure 5B). No other statistically significant differences were noted in baseline secretion of cytokines from PBMCs in culture after 3 h (Figures 5C,D) or 24 h (Figure 6) in culture, including CXCL1, IL-10, IFN-γ, IL-5, and IL-1β. Together, these results indicate that the PBMC secretome at

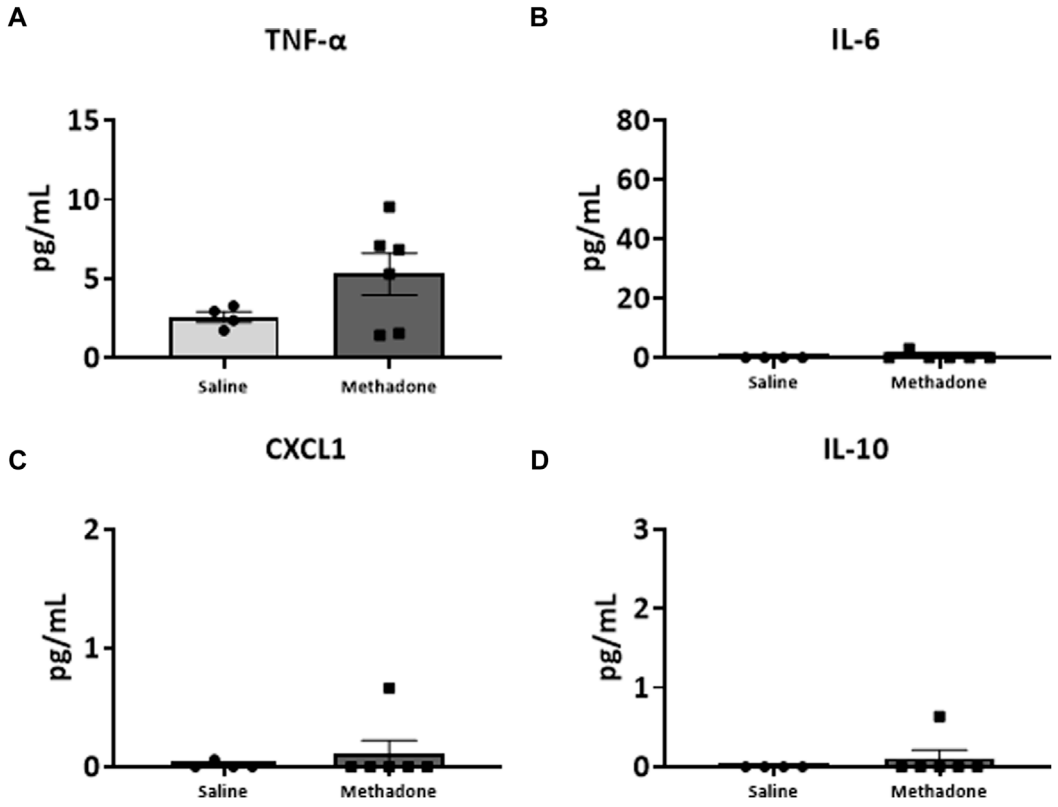

FIGURE 6
Alterations in baseline PBMC secretome are not observed after 24 h in culture. PBMCs were isolated from P21 pups prenatally exposed to saline or methadone. Conditioned media was then assessed for cytokines and chemokines after 24 h in culture. There were no significant differences in cytokine and chemokine levels between rats with POE and control animals (t-test for all).

baseline in P21 rats with POE is altered and favors a pro-inflammatory microenvironment.

POE induces sustained peripheral immune hyper-reactivity (SPIHR)

We next assessed the reactivity of PBMCs following a secondary immune challenge performed with LPS (n = 4/group for saline; 2 males; 2 females, n = 6/group; 3 males; 3 females for methadone). This was necessary to unmask characteristics of PBMC responsiveness following a secondary insult. There were significant elevations in TNF-α levels in PBMCs isolated from P21 rats with POE after LPS challenge at both 3h and 24 h in culture. Specifically, there was a notable 2.5-fold increase in TNF-α levels in rats with POE compared to controls following LPS stimulation at 3 h (saline: 69.68 ± 18.42 pg/ml, methadone: 175.9 ± 22.0 pg/ml, t-test, $p < 0.01$) (Figure 7A). There was also a more than three-fold increase in CXCL1 levels in rats with POE compared to controls after 3 h in culture with LPS (saline: 6.226 ± 0.65 pg/ml, methadone: 20.68 ± 11.6 pg/ml, t-test, $p < 0.05$) (Figure 7C). These increases in TNF-α and CXCL1 levels in the PBMC secretome of rats with POE was also seen after 24 h in culture with an almost 2-fold increase in TNF-α levels (saline: 193.4 ± 47.2 pg/ml, methadone: 360.6 ± 30.8 pg/ml, t-test, $p < 0.01$) (Figure 8A), and a 2-fold increase in CXCL1 levels (saline: 48.89 ± 18.07 pg/ml, methadone: 100.7 ± 12.3 pg/ml, t-test, $p < 0.05$) (Figure 8C). Following secondary immune challenge with LPS, the PBMC secretome in rats with POE demonstrates SPIHR with significant elevation in TNF-α and CXCL1 after both 3 and 24 h in culture.

Peripheral immune reactivity and hypercytokinemia normalize by P60

After assessing peripheral immune reactivity and the inflammatory profile at P21, we expanded our assessment by investigating the PBMC secretome at baseline and with LPS challenge from rats with POE and controls at P60 (n = 5/group for saline (2 males; 3 females), n = 7/group (4 males; 3 females) for methadone). Overall, individual cytokine and chemokine

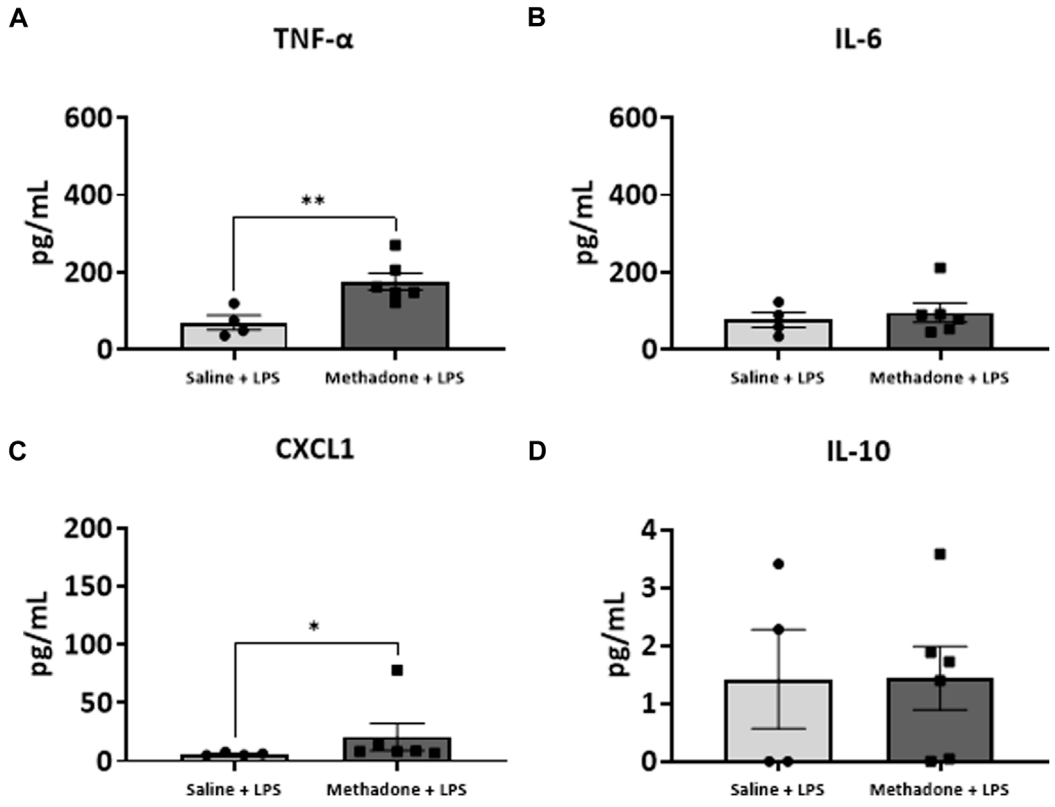

FIGURE 7
POE induces sustained peripheral immune hyper-reactivity (SPIHR) after 3 h in culture. PBMCs were isolated from P21 with POE. Conditioned media was then assessed for cytokine and chemokine levels after PBMCs were in culture for 3 h and following challenge with LPS. PBMC secretome from rats with POE demonstrated significant elevation in TNF-α levels compared to controls when stimulated with LPS (t-test for all, $*p < 0.05$, $**p < 0.01$).

expression levels were lower than the levels noted at earlier timepoints and developmental stages. There were no significant differences in any of the cytokine or chemokine levels including TNF-α, IL-6, CXCL1, IL-10, IFN-γ, IL-5, and IL-1β at baseline between POE and controls after both 3 and 24 h in culture ($p > 0.05$ for all, t-tests, data not shown). Following stimulation with LPS, cytokine and chemokine levels increased but again remained similar between the methadone-exposed and saline-exposed groups with no significant differences found after both 3 and 24 h in culture ($p > 0.05$ for all, t-tests, data not shown).

Discussion

Given the continued rise in opioid use across the globe, there is an urgent need to address the evolving public health crisis affecting countless pregnant women and children exposed to opioid medications *in utero*. Alongside growing recognition of the long-term neurologic impact associated with *in utero* opioid exposure, an improved understanding of the mechanisms underlying the brain injury caused by POE is imperative to establish biomarkers for identification of injury and to determine novel therapeutic targets. POE occurs at a critical timepoint in development disrupting delicate pathways essential for proper maturation of neural-immune function. Opioids readily cross the placenta and blood-brain barrier and lead to direct stimulation of inflammatory pathways *via* TLR4-mediated signaling (41–43). By shifting these pathways towards a pro-inflammatory state, opioids alter the developing immune system, and this alteration is sustained throughout the lifespan (2, 17, 44).

In this study, we show shifts in cerebral immune cell populations, defined specifically by increased neutrophils and regulatory T-cells, occurring months after prenatal opioid exposure. Furthermore, we demonstrate evidence of peripheral inflammation alongside immune priming and sustained peripheral immune reactivity (SPIHR) following prenatal opioid exposure that extends beyond the neonatal period. Even as markers of serum inflammation and SPIHR normalize into adulthood, elevated cerebral neutrophil and regulatory T-cell levels remain, highlighting the long-term impact of prenatal opioid exposure on the brain.

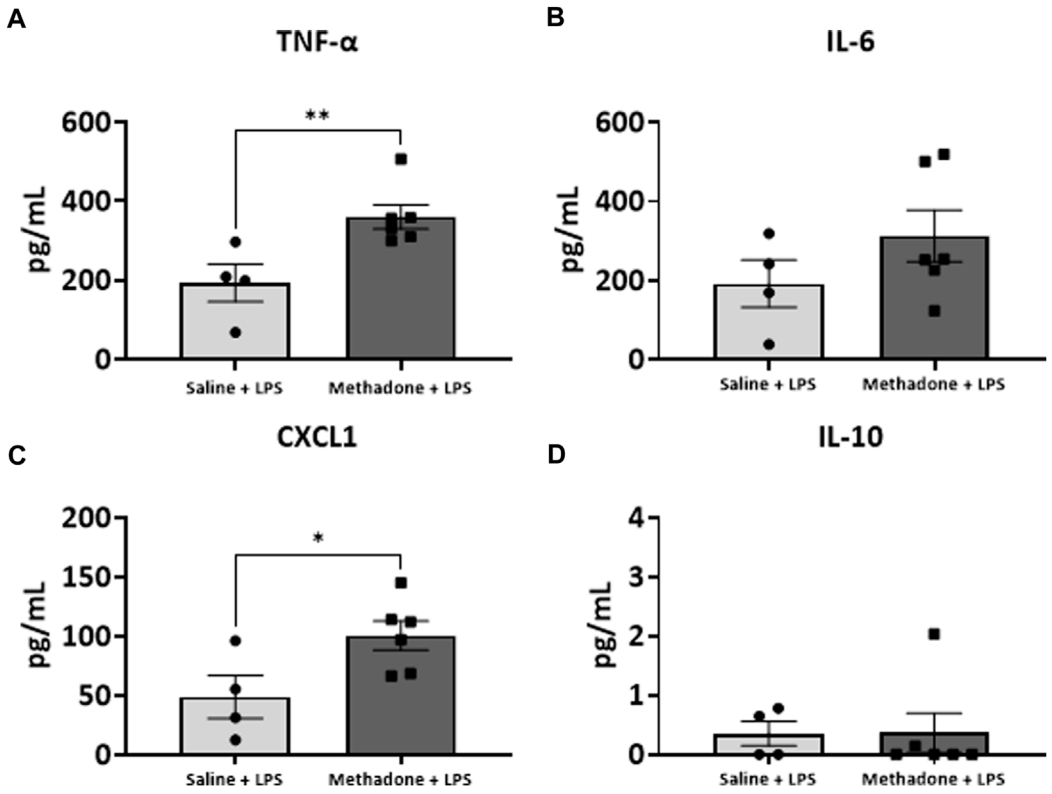

FIGURE 8
POE induces SPIHR after 24 h in culture. PBMCs were isolated from P21 pups. Conditioned media was then assessed for cytokine and chemokine levels after 24 h in culture following challenge with LPS. PBMC secretome from rats with POE demonstrated significant elevation in TNF-α and CXCL1 levels compared to controls (*$p < 0.05$, **$p < 0.01$).

Understanding the precise mechanisms underlying this injury is crucial to identifying those children at high risk of injury and to identifying targets for neuroimmunomodulation.

Previously, using the same model of POE, we identified a robust systemic inflammatory response syndrome and immune system dysfunction during the neonatal period concomitant with microstructural white matter injury and cognitive deficits in adulthood (17). POE led to priming of the immune system in the immediate perinatal period with significant baseline elevation in secretion of pro-inflammatory cytokines and chemokines, as well as an exaggerated inflammatory response from PBMCs acutely after stimulation with LPS (25). Specifically, we found of significant elevation of the inflammatory cytokines IL-1β, TNF-α, IL-6, and CXCL1 in the peripheral circulation at P10, around human term age equivalent, in POE rats suggesting a systemic inflammatory response syndrome (SIRS)-like response. We also showed that PBMCs, at P7, demonstrate significant baseline hypersecretion of TNF-α, CXCL1, and IL-6 with decreased levels of anti-inflammatory interleukin-10 (IL-10) and an exaggerated response to LPS stimulation with increased levels of TNF-α, CXCL1, IL-6, and IL-10 in rats with POE compared to controls (17, 25). Here, we extend those finding to show increased serum CXCL1 and IL-6 at P21, in POE rats concomitant with pro-inflammatory, dynamic, PBMC reactivity at P21, toddler age equivalent. CXCL1 is a potent chemokine responsible for neutrophil chemotaxis that has been implicated in significant intrauterine, placental, and fetal inflammation secondary to chorioamnionitis (26, 27, 33). Dysregulation in peripheral cytokine levels is not unique to POE and has also been identified in children with neonatal encephalopathy, cerebral palsy, and trisomy 21 (45–48). Our PBMC data reveals baseline hypersecretion of IL-6 that persists at P21. Further interrogation of PBMCs in culture with LPS stimulation at P21 demonstrates dysregulation in immune response following POE. After a 3 and 24 h incubation period, we note hypersecretion of TNF-α and CXCL1 in methadone-exposed PBMCs compared to saline-exposed PBMCs. These alterations in the baseline immune system function and immune response are indicative of SPIHR. The implications of inflammation during this timeframe from the neonatal period into toddler age equivalent cannot be understated, as it coincides with the

elaborate neurodevelopmental program guiding myelination, oligodendrocyte maturation, and neural circuit formation that remains vulnerable to disruption (49, 50). Taken together with emerging clinical literature supporting priming of the immune system secondary to opioid exposure *in utero*, these data support a unique inflammatory signature followed by increased sensitivity to future insults. This altered immune landscape may lead to aberrant neural maturation and long-term cognitive and functional impairment that is just starting to be recognized in countless children and adults who have been exposed to opioid medications prenatally and has been demonstrated in elegant preclinical studies (15, 19–23).This suggests important future directions to identify correlations with severity of brain injury with functional magnetic resonance imaging, longitudinal assessment of white matter injury, and assessment of additional domains of cognition, including attention and inhibitory control using the touchscreen platform.

To our knowledge, this is the first investigation highlighting changes in cerebral immune cell populations into adulthood following opioid exposure commencing *in utero*. Our data shows persistently abnormal populations of cerebral lymphocytes following POE, with increased regulatory T cells and neutrophils compared to control rats. This finding offers invaluable insight into long-term immune alterations that coincides with long-term deficits seen in individuals with prenatal opioid exposure (51–53). It also underscores the need for improved biomarker development to better identify those at risk of persistent inflammation into adulthood including imaging or functional assessments of injury. Furthermore, increased regulatory T cells in the brain is consistent with other models of perinatal brain injury that show tissue injury, neuronal loss, and abnormal long-term neurodevelopment associated with increases in T cells (54). Indeed, modulation of regulatory T cells may be neuroprotective under specific conditions of developmental brain injury and offers an important therapeutic avenue for neuroimmunomodulation to treat brain injury and neuroinflammation secondary to POE (54). Serum elevations of IL-6 and CXCL1, as well as hypersecretion of TNF-α and CXCL1 following LPS stimulation of PBMCs in rats with POE at P21 may also serve as valuable markers of earlier dysfunction in the toddler age equivalent that could suggest risk for future persistent immune alterations. This is evident as CXCL1, a potent chemokine in neutrophil chemotaxis, is upregulated after POE and exerts a lasting impact on cerebral inflammation with a persistent increase of neutrophils months after opioid exposure. Promoting homeostasis, rather than antagonism of these unbalanced inflammatory pathways, may be crucial in the treatment of brain injury secondary to POE due to key neurodevelopmental roles of both CXCL1 and TNF-α.

Critically, our data in rats with POE also suggests that the period in which to intervene, from a clinical perspective, extends beyond the neonatal period. While ongoing inflammation, detected even months after cessation of exposure to opioids, can negatively impact brain function into adulthood (44), it also potentially broadens the timeframe for intervention. Targeting persistent inflammation into toddlerhood may still influence long-term developmental outcomes in this high-risk population. Further investigation of changes in immune cell populations in the peripheral circulation using flow cytometric analyses across a similar time course beginning at P21 may offer insight into the specific pattern of inflammation and immune dysregulation associated with POE and provide guidance for discovery of earlier biomarkers, especially in discrete developmental subsets. Additionally, analysis of central immune cell populations in animals, including inflammatory activation and morphology assessments, may help characterize the impact of these alterations on the developing and aging CNS.

There are limitations to the design and scope of our study and future investigations will address these constraints. Firstly, our study was not powered to evaluate differences in outcome measures based on sex. Further investigation into sex as a modifier of inflammation secondary to POE is important to identifying at-risk infants and children and evaluating responsiveness to novel therapeutic approaches including neuroimmunomodulation. Second, opioid exposure in this model occurs from E16 through P21 and does not capture opioid exposure from the onset of pregnancy (E0). Last, a brief period of isoflurane anesthesia was administered during the third trimester of rat gestation to implant osmotic minipumps, and this may have been an additional inflammatory stimulus during pregnancy. Future studies will address the correlation between immune function, functional and structural brain injury, and deficits of cognition and attention in adulthood. Longitudinal assessment of inflammation and the immune system following POE in the same rat, over time would be beneficial.

In conclusion, we provide evidence of peripheral inflammation alongside immune hyper-reactivity following prenatal opioid exposure. The importance of neural-immune communication and crosstalk with the peripheral immune system and central immune system is further highlighted with durable changes in cerebral immune cell populations of regulatory T cells and neutrophils months after POE. Beyond molecular inflammation, this study demonstrates immune cell population changes in adulthood secondary to prenatal exposure that may be critical to understanding the underpinnings of injury associated with opioid exposure. This study adds to a growing body of important literature linking sustained changes in immune reactivity with developmental brain injury (45–48). Furthermore, this study offers insight into potential treatment targets and widens the time course for potential intervention to help countless children with brain injury resulting from prenatal opioid exposure.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Johns Hopkins University Animal Care and Use Committee.

Author contributions

Conceptualization and design, LJ; methodology, YK, MO, NM, SH, SRo, and LJ; investigation, NM, YK, SH, SK, RS, SRa, SM, and VV; formal analysis, NM, YK, SRo, GG, MO, and LJ; writing—original draft preparation, NM, GG, MO, SRo, and LJ; writing—review and editing, all authors; supervision, project administration, funding acquisition, correspondence and material requests, LJ. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Corrigendum: Methadone alters the peripheral inflammatory and central immune landscape following prenatal exposure in rats

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The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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