Title Genetic, Epigenetic, and Environmental Influences on Fetal Alcohol Spectrum Disorder: Implications for Diagnosis, Research and Clinical Practice

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Abstract
Fetal Alcohol Spectrum Disorder (FASD) is a diagnostic term in the Canadian guidelines and an umbrella term in the US guidelines, that describes the broad range of adverse effects that can result from prenatal exposure to alcohol, including deficits across multiple physical, physiological, neurobiological, and behavioral domains. The prevalence of FASD is estimated to be 1.1-5.0%, which is significantly higher than that of other common disorders, including Autism Spectrum Disorder and Down Syndrome. This relatively high prevalence highlights the urgent need for better recognition, diagnosis, and treatment of this disorder. Currently, however, there are numerous challenges in obtaining an accurate and reliable diagnosis of FASD, and many children remain undiagnosed. This dilemma further highlights the need for new screening and diagnostic tools that provide sensitive biomarkers of prenatal alcohol exposure. Identification of at-risk children at a young age will allow these children access to early interventions and services, which can profoundly change the long-term outcomes and quality of life of individuals with FASD and their families. In this Chapter, we discuss the evidence supporting the emerging potential for genetic, epigenetic, transcriptomic, and proteomic approaches to elucidate FASD etiology, and to serve as potential biomarkers or signatures of early-life events, including prenatal alcohol exposure. We provide an overview of FASD and a brief history of the development of diagnostic criteria, review risk and resilience factors that impact expression of the disorder, discuss genetic and epigenetic factors in FASD, and conclude by relating these findings to the clinical context.

Key words: prenatal alcohol exposure, Fetal Alcohol Spectrum Disorder (FASD), biomarkers, risk and resilience, genetics, epigenetics, transcriptomics and proteomics

Introduction

The adverse effects of prenatal alcohol exposure on offspring development were first described in papers by Lemoine and colleagues [1] and then by Jones and colleagues [2], who coined the term Fetal Alcohol Syndrome (FAS). The key observations by both groups included pre- and post-natal growth deficiencies, minor facial abnormalities, and damage to the developing brain that could result in behavioral, cognitive, attention, and adaptive alterations. Since then, thousands of clinical studies have confirmed and considerably extended these initial findings, describing deficits across multiple physical, physiological and neurobiological domains [3–5]. The term fetal alcohol spectrum disorder (FASD) was first introduced around the year 2000 to recognize more specifically this broad spectrum of effects, and was formalized in April 2004 when experts from several organizations (National Organization on Fetal Alcohol Syndrome, National Institutes of Health, Center for Disease Control and Prevention, and Substance Abuse and Mental Health Services Administration) developed a consensus definition of FASD [6]: "FASD is an umbrella term describing the range of effects that can occur in an individual whose mother drank alcohol during pregnancy," noting that, "These effects include physical, mental, behavioral, and (or) learning disabilities with possible lifelong implications".

Despite this wealth of evidence of alcohol's adverse effects, there are numerous challenges in obtaining an accurate and reliable diagnosis of FASD [7]. Characteristic facial abnormalities associated with FAS may not be present, or if present, can change with age. Environmental influences can impact physical, cognitive and behavioral problems. It is often difficult if not impossible to obtain an accurate history of maternal alcohol consumption. Moreover, independent of whether facial abnormalities are present, differentiation of FASD from other disorders with overlapping physical features and/or neurobehavioral deficits can be challenging. It is increasingly evident that new diagnostic tools that can provide sensitive biomarkers of prenatal alcohol exposure and that can be ethically applied would make a significant impact in the diagnosis and prevention of FASD. It is well established that early cognitive, educational, adaptive, and behavioral interventions can profoundly change the long-term outcomes and quality of life of these individuals and their families [8–10]. Knowing this impact, early screening tools are essential to help identify at-risk children at a young age and provide an objective clinical assessment that will allow these children access to early interventions and services. In this Chapter, we discuss the evidence supporting the emerging potential for genetic, epigenetic, transcriptomic and proteomic approaches to elucidate further FASD etiology, and to serve as potential biomarkers or signatures of early-life events, including prenatal alcohol exposure. We provide an overview of FASD and a brief history of the

development of diagnostic criteria, review risk and resilience factors that impact expression of the disorder, discuss genetic and epigenetic factors in FASD, and conclude by relating these findings to the clinical context.

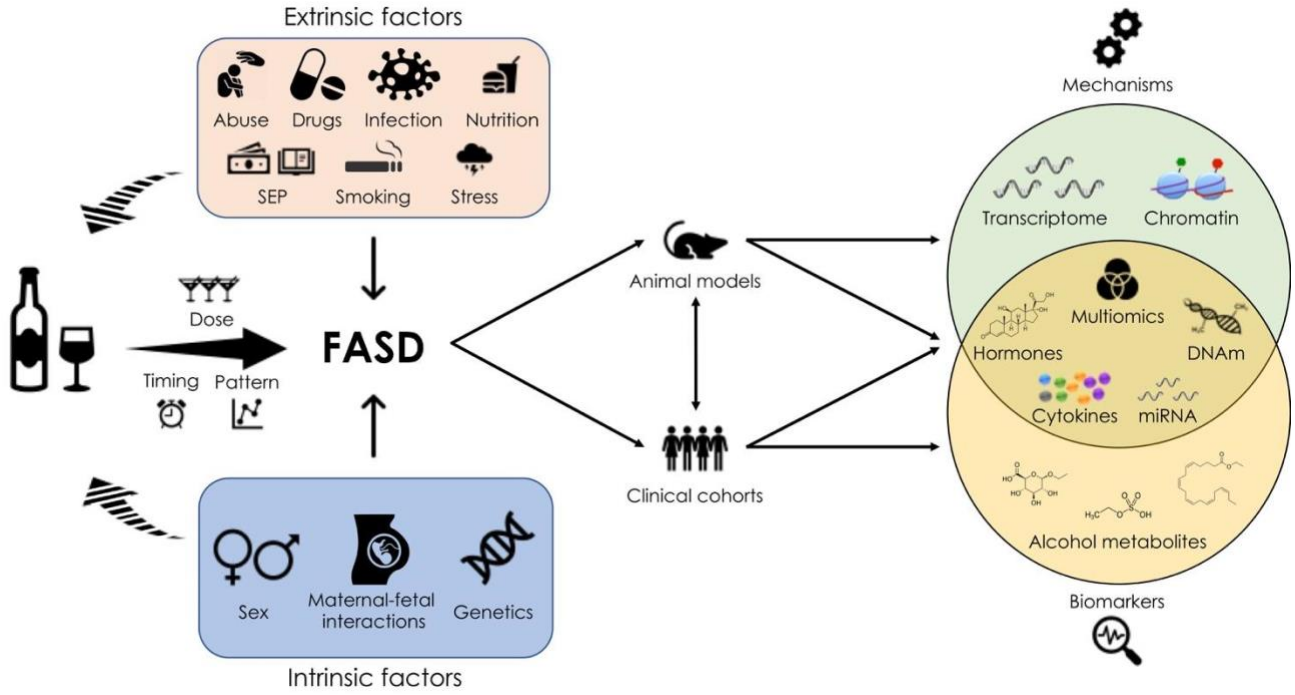

Figure 1. An overarching framework of fetal alcohol spectrum disorder. Several factors influence the manifestation of fetal alcohol spectrum disorder (FASD), beginning with the pattern, timing, and dose of prenatal alcohol exposure. Extrinsic and intrinsic factors can also exert their influence at multiple stages, including both the prenatal period and during postnatal development. These factors are studied in two basic approaches, animal models and clinical cohorts. Both approaches provide insight into the mechanisms driving the effects of alcohol on development and aid in the development of biomarkers of FASD. Finally, some types of analyses may be better suited than others for mechanistic insights or the development of biomarkers, although many show a broad range of applicability. DNAm = DNA methylation; SEP = Socioeconomic position.

Section 1. FASD as a common neurodevelopmental disorder: prevalence and diagnosis

Until recently, the prevalence of FASD was estimated at approximately 10 per 1000 children, using clinic-based studies or studies of individual communities [11]. However, it is now known that this is likely an underestimate of the true prevalence. Many children with FASD remain undiagnosed or are misdiagnosed; among other reasons, this might occur when trained dysmorphologists are not available to make a diagnosis or when individuals present with neurobehavioral deficits in the absence of dysmorphic features, and maternal drinking history is unknown. A recent study utilizing active-case ascertainment and a cross-sectional design to assess first graders at four community sites reported an

estimated prevalence of FASD ranging from 1.1% to 5.0% [11]. While these findings may not be generalizable to all communities, these data suggest that the prevalence of FASD is likely higher than previously thought. Moreover, the prevalence of FASD is significantly higher than that of other common disorders, including Autism Spectrum Disorder, estimated at 2.20%-3.31% in 2016 [12], and Down Syndrome, estimated at approximately 1 in 1,499 (or 0.67%) in the USA [13]. The relatively high prevalence of FASD highlights the urgent need for better recognition, diagnosis, and treatment strategies.

A variety of diagnostic guidelines have been developed over the years to assist clinicians in recognizing and diagnosing children exposed to alcohol *in utero* (reviewed in [7]). Following the identification of FAS, the term suspected fetal alcohol effects (FAE) [14] was introduced to describe partial expression of FAS, where some but not all of the features seen in FAS are present, and recognizing that a range of deficits can occur, even in the absence of obvious facial dysmorphology. In 1996, an Institute of Medicine (IOM) committee identified four alcohol-related clinical diagnostic categories [15]: 1) Fetal alcohol syndrome (FAS: evidence of characteristic craniofacial dysmorphology, prenatal and postnatal growth restriction, and CNS neurodevelopmental deficits); 2) Partial fetal alcohol syndrome (pFAS: some but not all of the characteristics features of FAS and confirmed maternal alcohol exposure); 3) Alcohol-related neurodevelopmental disorder (ARND: evidence of CNS neurodevelopmental abnormalities and (or) of a complex pattern of behavioral or cognitive abnormalities, with confirmed maternal alcohol exposure); and 4) alcohol-related birth defects (ARBD: One or more congenital anomalies and confirmed maternal alcohol exposure). Categories 3 and 4 are not mutually exclusive. To "operationalize" the IOM criteria, Astley and Clarren [16] developed the FASD 4-digit diagnostic code, utilizing a Likert scale (1 = absence of the feature, 4 = strong presentation of the feature) to reflect magnitude of expression of each of: growth deficiency, the FAS facial phenotype, CNS dysfunction, and gestational exposure to alcohol. Updated versions of this system are now widely used.

Updated clinical guidelines for FASD have been published over the last 10-15 years, in general building on the IOM criteria and 4-digit code, and include those by Hoyme and colleagues [17], and two sets of Canadian Guidelines [18,19]. Of note, the 2016 revision of the Canadian Guidelines made FASD a diagnostic term and collapsed the diagnostic categories to two: (*i*) FASD with sentinel facial features and evidence of impairment in 3 or more identified neurodevelopmental domains, with prenatal exposure to alcohol either confirmed or unknown and (*ii*) FASD without sentinel facial features, with evidence of impairment in 3 or more identified neurodevelopmental domains, and confirmed prenatal exposure to

alcohol. These Guidelines also specify a category called "At risk for neurodevelopmental disorder and FASD, associated with prenatal alcohol exposure," which may help to identify individuals who are at risk. Most recently, the Diagnostic and Statistical Manual (DSM), 5th edition, from the American Psychiatric Association, includes "neurobehavioral disorder associated with prenatal alcohol exposure (ND-PAE)" (American Psychiatric Association 2013) under the "Conditions for Further Study" and also given as an example under "Other Specified Neurodevelopmental Disorder (315.8)". ND-PAE allows clinical assessment that encompasses the neurodevelopmental and mental health issues associated with prenatal alcohol exposure[20]; it can be diagnosed in either the presence or absence of physical effects of prenatal alcohol exposure, requires confirmed gestational exposure to alcohol, and includes symptoms in three broad domains - neurocognitive functioning, self-regulation, and adaptive functioning - that adversely impact quality of life. Inclusion of FASD in the DSM is a critical step forward in both bringing FASD to the attention of clinicians and increasing access to services for individuals exposed to alcohol *in utero*.

Section 2. Risk and protective factors for FASD – what is the evidence?

Following the identification of FAS, research in both human and animal studies blossomed, providing definitive evidence linking prenatal exposure to alcohol with FAS. Initially, this link was interpreted in a fairly straightforward manner, as reflected in the first Surgeon General's warning (U.S. Surgeon General 1981, p. 9): "The Surgeon General advises women who are pregnant (or considering pregnancy) not to drink alcoholic beverages and to be aware of the alcoholic content of foods and drugs." However, no specific guidelines were proposed, as outcomes were highly variable, and little was understood of the relationship between level of alcohol intake and severity of outcome.

In an attempt to understand both risk and protective factors, researchers initially focused on assessment of quantity, frequency, pattern, timing, and duration of alcohol consumption. Studies have consistently found a positive correlation between maternal blood alcohol concentration and developmental alterations, including physical, physiological, neurobiological, cognitive, and behavioral deficits [21–24]. However, the greatest risk is associated with heavy episodic or binge drinking, which results in the highest blood alcohol levels [25–27]. In that regard, it became apparent that measures of average consumption levels (*e.g.*, average drinks per day or per month) may not be useful and in fact may be misleading. Rather, assessment of the number of drinks per occasion, or measures such as maximum drinks per occasion or number of "binges" are more relevant for understanding fetal exposure and risk

in humans. The critical roles of timing and duration of exposure in risk are also well documented [28]. Organs and systems in the embryo are particularly vulnerable to structural and/or functional abnormalities during their period of most rapid growth and development. Two of the most vulnerable periods for brain development, for example, are the first half of the first trimester and the brain growth spurt that occurs in the third trimester [29]. Nevertheless, prenatal alcohol exposure can impair brain development during all stages of gestation, including effects on neurogenesis, differentiation, and synaptogenesis [28–31], as the brain develops over the entire gestational period and into postnatal life. Moreover, data suggest that variables such as quantity, frequency, pattern, timing, and duration of alcohol consumption cannot fully explain fetal or child outcomes. Increasing evidence suggests that numerous biological and environmental variables can further exacerbate risk or act as protective factors [10,15,25–27].

Maternal and environmental factors:

Body profile, age, gravidity, parity, metabolism. Maternal body profile is associated with birth outcomes: lower than average height, weight and BMI are more often seen in women who have children with FASD than in those whose children are unaffected [26]. This may reflect undernutrition in these women, or possibly, that some of these women themselves may have FASD. Average risk of having an affected child and/or a more severely affected child is also increased in women who are older and who have higher gravidity (more pregnancies) and parity (more children) compared with the risk for younger women who drink at similar levels. Women who have children with FASD also have more miscarriages and stillbirths [25,26]. The reason for these associations is not entirely clear, but may be related to severity of addiction, such that these women cannot decrease their alcohol intake during pregnancy. Continued drinking over time may also cause deterioration of health and nutritional status. In particular, May and colleagues [32] reported that an early age of initiating regular drinking may exacerbate the adverse effects of alcohol by increasing the amount of time that alcohol can affect biological and physiological processes important for alcohol metabolism. These effects can vary from one individual to the next and within individuals depending on nutrition, body weight, and physiological factors, as well as genetic and environmental influences [26].

Nutrition. Nutritional deficiencies have been linked to increased risk for adverse birth outcomes in general [25]. Importantly, alcohol intake can directly affect nutrient intake, resulting in primary malnutrition or undernutrition [33]. Because of its high energy value (7.1 kcal/g), alcohol may displace other food in the diet; as calories provided by alcohol are not associated with essential nutrients, intake

of these “empty” calories can result in nutritional deficiencies. This is particularly problematic during pregnancy and lactation when nutrient requirements are considerably (10-30%) higher than in non-pregnant/non-lactating females. Economic issues may also play a role if a portion of the food budget is spent on alcohol in place of nutritious foods.

Secondary undernutrition is also an issue [33,34], as the deleterious effects of alcohol occur in the placenta and at virtually every level of the gastrointestinal tract, resulting in altered metabolism, transport, utilization, activation, and storage of most essential nutrients. For example, effects on the umbilical circulation and on blood flow to the placenta will reduce oxygen supply as well as transport of essential nutrients to the fetus. The most commonly reported nutritional deficiencies and their major effects are shown in **Table 1** [33–35].

Table 1. Nutritional Deficiencies and Related FASD Effects

Nutrients	Major effects
Protein/amino acids	Decreased organ growth and development, brain damage
Thiamin [vitamin B1]	Cardiovascular and nervous system function
Riboflavin [vitamin B2]	Activation of anti-oxidant enzymes
Vitamins B6 and B12	Cellular function, neurotransmitter synthesis, metabolism of glucose, lipids, proteins, alcohol
Vitamin E	Anti-oxidant, stability of free radicals
Selenium	Cofactor for antioxidant enzymes, thyroid hormones
Vitamin A	Organogenesis, cell and neuronal growth and differentiation
Vitamin C	Anti-oxidant
Folic acid	Premature birth, fetal malformations [eye, palate, GI tract, kidneys, skeleton, nervous system (including neural tube defects)]
Vitamin D and zinc	DNA, RNA stability, preterm delivery, increased incidence of birth defects
Choline	alcohol-induced alterations of the hippocampus and prefrontal cortex (PFC)
DH3 [Omega-3 fatty acids]	antioxidant mechanisms in the brain and liver

Socioeconomic Position (SEP). SEP is a major determinant of health that encompasses a broad range of social and economic factors, such as income, education, occupation level, etc, that can represent health inequalities or influence an individual’s health outcomes[36]. Of note, SEP is used here instead of the commonly used ‘socioeconomic status’ (SES), as the latter does not distinguish between actual resources and the status or rank-related characteristics of socioeconomic factors[36]. Although children with FASD have been identified in all SEP groups, data from several countries and epidemiological studies suggest that FASD may be more frequent in individuals in lower SEP categories [27,37]. For example, quantity of

alcohol consumed prior to knowledge of pregnancy, Total Distress score and SEP, taken together, were more highly associated with diagnosis of an FASD than quantity of alcohol consumed [38]. Associations between low SEP and FASD likely stem from findings that lower SEP may be associated with poor living conditions, poor nutrition, lower levels of education and employment, and high levels of stress, which are all associated with poorer birth outcomes. On average, infants born to women in lower SEP conditions have lower birth weight and length, smaller heads, more malformations, and higher levels of attention deficit disorder, whether alcohol-exposed or not [27,37]. Studies also suggest that the severity of FASD effects is influenced or modulated by the stability and nurturing of the postnatal environment, which are also associated with SEP and maternal education, as well as marital and employment status [25].

Stress. Stress can be defined as a state of threatened homeostasis or internal steady state [39]. Stressors, both physical and psychological, can disturb homeostasis and activate a set of adaptive responses that enable the individual to respond to and cope with the stressors and thus restore homeostasis. The two key components of the stress system are the autonomic nervous system that initiates a rapid "fight or flight" response, and the hypothalamic-pituitary-adrenal (HPA) system that initiates a slower and longer lasting hormonal response. Here we will focus primarily on the HPA axis due to its extensive and long-lasting effects on the body as well as its relevance for FASD. The HPA axis comprises a cascade of responses, ultimately resulting in the release of the stress hormone cortisol, which affects virtually every system in the body and facilitates coping and the restoration of homeostasis. In the short term, HPA activation is helpful, mobilizing energy, increasing cardiovascular tone and circulating glucose levels, and suppressing responses not immediately necessary for coping, including digestion and the immune response. In the long-term, however, chronic stress and chronically high levels of HPA activity/hormones, will have adverse consequences including fatigue, hypertension, ulceration, metabolic alterations, vulnerability to infections or diseases, and even neuron death.

Not surprisingly, exposure to stressors during pregnancy can have adverse effects on pregnancy outcome, maternal and fetal health, and offspring behavioral, immune, cognitive, and physiological development [40,41]. Maternal stress has a marked negative impact on maternal endocrine and immune systems, which interact with each other and with the central nervous system in an intimate bidirectional manner. Alterations in activity and function of any one of these systems will affect the others, and insults that alter activity of these systems in the mother will affect the development of fetal metabolic, physiological, endocrine, and immune function, with potential long-term consequences for development and health.

Importantly, alcohol consumption during pregnancy can disrupt the normal hormonal interactions between the pregnant female and fetal systems, altering the normal hormone balance, including activity of the HPA axis. Compounding the effect of prenatal alcohol exposure, children with FASD often experience a high level of early life adversity (*e.g.*, maltreatment, early caregiving disruption and contact with the foster care system, poverty, and familial adversity) [10,42,43]. The adverse effects of early life adversity can parallel in some ways the adverse effects of prenatal alcohol exposure, particularly on physiological and behavioral outcomes. In the human situation, however, it is difficult if not impossible to separate the effects of prenatal alcohol exposure from those of early life adversity, and studies evaluating the impact of prenatal alcohol may, at least in some cases, be studying both prenatal alcohol and environmental stress/adversity and/or their interactions.

Paternal factors:

In contrast to the large body of research on the influence of maternal factors on offspring outcome, much less attention has been paid to the possible role of preconceptional paternal factors. However, data indicate that a large proportion of women who drink alcohol associate with men who also drink alcohol [44]. Therefore, it is possible that at least some of the abnormalities attributed to the teratogenic effects of maternal drinking may be related to or exacerbated by paternal drinking. While evidence for the importance of paternal factors has emerged, the mechanisms responsible are not yet well understood [25,44,45]. As alcohol contains toxins that can impact health, for men who use alcohol and father children, their semen may contain toxins that could damage the DNA of the fetus [45]. Moreover, both epidemiological and laboratory studies have shown genetic and epigenetic alterations that may underlie offspring outcomes following both maternal and paternal alcohol intake [46].

Section 3. Genetics of FASD

Although FASD is essentially an environmental disorder, twin studies have shown that genetic mechanisms may play a role in resilience or vulnerability to the effects of alcohol exposure *in utero*. In one study, for example, while identical twins were 100% concordant for a specific FASD diagnosis, fraternal twins showed 56% concordance [47]. Of these, four pairs of fraternal twins had divergent diagnoses - partial FAS versus neurobehavioral disorder/alcohol exposed – despite sharing 50% of their genetic information and presumably receiving virtually identical alcohol exposure. By contrast, full siblings who also share ~50% of their genomes, but may have different levels of alcohol exposure, show

only 41% diagnosis concordance. These findings suggest that while fetal genetics likely play a key role in mediating the effects of alcohol exposure during embryonic development, environmental factors are also likely involved.

Here, we examine the underlying genetic mechanisms that may predispose and further exacerbate the effects of alcohol in animal models and humans, alike. To this end, we will discuss gene networks that may be involved in the development of the FASD sentinel facial features and potential comorbidities. Of note, the majority of these pathways have been identified through work in animal models, including but not limited to *Xenopus*, zebrafish, chicks, and mice, as these models allow for direct manipulation of genetic pathways and controlled alcohol exposure.

Genes and Cell Signaling Pathways involved in FASD:

Numerous pathways and signaling cascades have been associated with the development of physical alterations reminiscent of the FASD sentinel features, including both genetic pathways (*i.e.* bone morphogenetic protein (BMP), fibroblast growth factor (FGF), sonic hedgehog (SHH), Wingless (WNT) and biochemical factors (*i.e.* folic acid, retinoic acid, hormones, etc.). In particular, these signaling molecules are crucial for proper craniofacial formation and mediate the genetic cross-talk necessary to form appropriate gradients and signaling pathway cascades during development. Furthermore, animal models have shown that genes within these networks and biochemical factors are altered by developmental alcohol exposure, potentially mediating the effects of alcohol on the developing organism, as outlined below. Importantly, both the direct influences of prenatal alcohol exposure on the expression of these genes or genetic variation within these pathways could lead to more or less vulnerability to the effects of alcohol.

SHH mutations and signaling pathway impairments in FASD

Sonic hedgehog (SHH) mutations are most commonly associated with craniofacial midline defects, such as holoprosencephaly and impairments in the frontonasal prominence and both maxillary and mandibular processes [48]. These craniofacial malformations are reminiscent of alcohol-induced craniofacial malformations across different species [49–51]. Importantly, it is known that alcohol activates direct antagonists of SHH signaling, such as the cAMP pathway and protein kinase A [52]. Furthermore, SHH and its respective pathways can be indirectly affected by anti-factor developmental morphogens such as

retinoic acid, FGF, BMP4, and transforming growth factor β-1 (TGFβ-1) family member genes, which disrupt SHH gradients in the primitive streak and developing neural floor plate during gastrulation [53].

Of note, biochemical cross-talk between the cholesterol and SHH pathways may contribute to craniofacial malformations through SHH signaling. Indeed, the SHH protein requires the addition of cholesterol and palmitate to become biologically active[54]. Without these modifications, the SHH protein cannot be transported out of the cell or bind to lipid rafts within the plasma membrane for transport and signaling transduction. In addition, cholesterol can bind to the *Smo* protein directly; *Smo* is an SHH ligand required for activation of the GliA pathway and target gene activation for proper midline formation [55]. Given that these pathways are also affected by alcohol exposure (see below), SHH signaling may act as a key integration pathway for the sentinel features of FASD.

WNT mutations and signaling pathway impairments in FASD

Wingless (WNT) signaling is implicated in many developmental processes, including proper mitogenic stimulation, as well as cell fate specification and differentiation [56]. Studies across different model organisms have shown that alcohol can impact the WNT pathway at multiple points, causing aberrant cell migration and cellular differentiation in the gastrulating embryo (reviewed in [57]). Alcohol can also indirectly trigger the WNT pathway, causing abnormal expression of gene targets required for differentiation, migration, and proliferation and ultimately leading to neural crest cell apoptosis [58]. Importantly, acute alcohol exposure can impair the WNT/β-catenin canonical signaling pathway, causing impairment of cartilage and bone formation, and neural crest cell lineage tissues [59]. Furthermore, disruption of cranial neural crest cell migration due to aberrant WNT signaling can result in impaired fusion of the nasal and maxillary processes, leading to cleft lip and/or cleft palate formation, which are the same craniofacial regions where key FASD sentinel features are found [60]. Taken together, this body of work indicates that the WNT pathway is a target of alcohol and may play a role in the development of FASD and its sentinel facial features.

FGF signaling pathway

Fibroblast growth factor (FGF) signaling is one of the most important signaling factors in the developing embryo, required for cellular proliferation, differentiation and migration, as well as proper axial development and craniofacial formation, specifically for ossification of cranial bones and suture homeostasis [61]. Given that several genes within this pathway are downregulated by prenatal alcohol in animal models, including *FGF2*, *FGF8,* and *FGFR2,* perturbation of this crucial craniofacial

development pathway by alcohol could potentially drive some of the sentinel features observed in FASD [52,62]. Of note, the FGF pathway also shows extensive crosstalk with the SHH, WNT, and retinoic acid pathways to control differentiation and patterning during development, suggesting that it may act as a focal point for genetic contributions to FASD [63].

BMP signaling pathway

Bone morphogenic protein (BMP) signaling is an important signaling factor in the developing embryo, required for cellular growth, differentiation and apoptosis. Similar to features seen in individuals with FASD, knock-out of BMP receptors impairs mesoderm and neural crest cell lineages, leading to lip, palate, and eye defects, as well as brain, cardiac, skeletal, and tooth defects [64]. Studies in both mouse models and clinical cohorts [65,66] have shown that alcohol exposure during gastrulation causes dysregulation of the BMP signaling cascade, leading to congenital heart defects. As such, it is possible that alterations in the BMP pathway may play a key role in mediating cardiac-related deficits in FASD.

Retinoic acid deficiency and signaling pathway impairments in FASD

Retinoic acid, the metabolized form of Vitamin A (retinol), is a crucial factor in development, playing key roles in craniofacial, cardiac, and limb development. Importantly, retinoic acid deficiency at early gastrulation has been shown to produce FASD-like craniofacial defects such as smaller eyes, microcephaly, reduced axial development, and a lack of the forebrain ventricles [67,68]. Furthermore, retinoic acid supplementation can partially rescue the effects of acute alcohol exposure [67,69]. Of note, acetaldehyde competes with retinaldehyde for aldehyde dehydrogenases, becoming a rate-limiting step of retinoic acid metabolism. As such, variation in these genes could impact the bioavailability of retinoic acid, leading to deleterious effects on the developing organism. Taken together, these data suggest that retinoic acid or Vitamin A deficiency may be an underlying factor in the etiology of FASD, further exacerbating the developmental effects of prenatal alcohol exposure during early development.

Susceptibility and resilience genes and factors in FASD:

Although research has revealed many of the cell signaling pathways impacted by alcohol during development, insights into genetic factors that may further predispose an individual to FASD are not well understood. Various maternal factors act to increase the risk of alcohol's deleterious effects in the developing fetus. In particular, the presence of genetic polymorphisms of alcohol-metabolizing enzymes may increase or decrease alcohol's deleterious effects. For example, maternal polymorphisms

manifesting as increased alcohol dehydrogenase activity and enhanced alcohol metabolism have been associated with a decreased incidence of alcohol teratogenicity [70,71], possibly by impacting the capacity of the maternal-fetal unit to metabolize alcohol. As the capacity for alcohol metabolism among pregnant women can vary up to eightfold (from 0.0025 to 0.02 g/dl/h), the variation in phenotypic presentation of FASD in women consuming similar doses of alcohol could be mediated, at least partly, by genetic mechanisms.

Co-morbidities with other developmental genetic disorders:

DiGeorge syndrome, also known as 22q11.2 deletion syndrome (22q11.2DS), is a sporadic autosomal dominant disorder caused by a 1.5-3 Mb microdeletion on chromosome 22 encompassing approximately 30 genes [72]. Interestingly, 22q11.2DS shares many craniofacial and cardiac malformations with FASD, CHARGE syndrome, and retinoic acid embryopathy, including long philtrum, thin upper lip, upward slanted palpebral fissures, and aortic arch abnormalities[73,74]. CHARGE syndrome refers to a set of developmental malformations that vary in their presentation, and include: ocular coloboma (C), heart disease (H), choanal atresia (A), retarded growth and/or anomalies of the central nervous system (R), genito-urinary defects and/ or hypogonadism (G), and ear anomalies and/or deafness (E)[75]. In a recent study, mutations in the CHD7 (Chromatin helicase DNA binding protein 7) gene, an evolutionarily conserved protein required for proper chromatin remodeling, were found in 83% of CHARGE syndrome patients[76]. CHARGE syndrome and 22q11.2DS brain aberrations such as reduced white matter tract volume and behavioral abnormalities such as attention deficit hyperactivity disorder (ADHD) are found in individuals with FASD and Autism Spectrum Disorder[76–79]. It is not a coincidence that these disorders share these malformations, as the malformations result from altered differentiation, proliferation, and migration of neural crest cells (NCC), which result in hindbrain, frontonasal prominence, and pharyngeal arch aberrations. Interestingly, Sulik *et al.* found that alcohol exposure in a mouse model at embryonic day 8.5 causes craniofacial malformations reminiscent of DiGeorge syndrome and VeloCardioFacial (Shprintzen) syndrome: micrognathia, low-set ears, abnormal pinnae, a short philtrum, midline clefts in the nose, cleft palate, and ocular hypertelorism [80]. T-Box Transcription Factor 1 (*TBX1*, a gene deleted within the 22q11 region) heterozygous mice can also phenocopy the craniofacial malformations found in DiGeorge Syndrome and in FASD [81]. Finally, while the outcomes are more severe, retinoic acid embryopathy and Vitamin A deficiency syndrome produce craniofacial and brain malformations and upper trunk anomalies (heart, lungs, thymus, thyroid, for example) reminiscent of those seen in FASD as well as other syndromes including DiGeorge and CHARGE syndrome.

Of note, FASD sentinel facial features can vary as they reflect outcomes of craniofacial development specific to the time of alcohol exposure. As developmental processes have exquisite temporal regulation, timing of the alcohol insult will differentially affect head, forebrain, and craniofacial development, contributing to the FASD spectrum. Nevertheless, the studies discussed above highlight the fact that genetic underpinnings of characteristic features of FASD suggest a powerful tool for further exploration of FASD etiology. Taken together, hemizygous expression of neural crest cell (NCC) genes such as *TBX1* could provide researchers an opportunity to use 22q11.2DS as a model to study NCC-related aberrations that occur in FASD. Moreover, harnessing whole genome sequencing, mutations and epigenetic modulation of modifier genes can be utilized to understand better the variable expressivity associated with 22q11.2DS and other NCC-related disorders, including the role of NCC alterations in disorders such as FASD and CHARGE syndrome. Future studies will allow better understanding of the role of developmental signaling pathways (SHH, FGF, BMP, retinoic acid) in FASD. Studies on the developmental signaling pathway defects shared with other disorders will expedite development of pharmacological and other interventions that might help to attenuate or ameliorate the malformations and co-morbidities associated with FASD and other disorders.

Section 4. Epigenetics of FASD

Although the exact molecular mechanisms underlying the effects of prenatal alcohol exposure on neurobiological systems are not yet fully elucidated, epigenetic mechanisms are prime candidates for the programming effects of environmental factors on physiological and neurobiological systems, as they may bridge environmental stimuli and neurodevelopmental outcomes to influence health and behavior well into adulthood. Epigenetics refers to modifications of DNA and/or its regulatory factors, including chromatin and non-coding RNA, that alter the accessibility of DNA to modulate gene expression and cellular functions without changes to underlying genomic sequences [82]. Patterns of epigenetic modifications, in general, have been closely associated with cell fate specification and differentiation, suggesting a crucial role for epigenetics in the regulation of cellular functions [83]. For a detailed overview of studies of DNA modifications and developmental alcohol exposure, please refer to [84].

DNA methylation:

DNA methylation is perhaps the most studied epigenetic modification and involves the covalent attachment of a methyl group to the 5’ position of cytosine, typically occurring at cytosine-guanine

dinucleotide (CpG) sites. In addition to its association with gene expression, it also plays a key role in the regulation of developmental programs [85]. DNA methylation is also emerging as a potential biomarker for early-life exposures due to its stability over time and malleability in response to environmental cues [86]. Numerous studies have identified changes in DNA modifications in response to prenatal alcohol exposure, ranging from "bulk" (or total) levels to candidate gene approaches and genome-wide associations. Early findings from animal models demonstrated that prenatal alcohol exposure appears to impair the establishment of DNA methylation levels primarily at the bulk level, suggestive of broader reprogramming of downstream cellular and biological functions. However, more recent studies have shown that rather than a global decrease in DNA methylation, some genomic regions show specific directions of effect [87]. These studies have attempted to identify specific genetic loci that may be more sensitive to alcohol-induced epigenetic alteration, using both hypothesis and discovery-driven approaches. As well, these studies have focused mainly on genes with known functions related to the deficits observed in FASD (immune, stress, cognition, etc.), identifying more specific changes to DNA methylation patterns. However, they have not been as successful in pinpointing new targets and mechanisms in the etiology of FASD. As such, current approaches are beginning to move beyond targeted analyses to assess the genome-wide effects of prenatal alcohol on the epigenome, without *a priori* hypotheses about which genes may be influenced. These approaches have yielded important insights into the broader molecular pathways involved in FASD pathogenesis, identifying new pathways and targets of alcohol-induced epigenetic alterations. Of note, recent studies have also used DNA methylation patterns as a potential biomarker of prenatal alcohol exposure. In animal models, our recent study showed that some DNA methylation patterns are concordant between the brain and blood of alcohol-exposed animals, suggesting that blood (white blood cells) may be an important surrogate tissue for the development of better FASD biomarkers [88], with critical implications for human studies. In humans, epigenetic biomarkers show promise for early screening of at-risk individuals, as the DNA methylome retains a lasting signature of gestational alcohol exposure in both the central nervous system and peripheral tissues (reviewed in [84]). Recently, DNA methylation profiles in a large cohort of children with FASD have established that epigenetic markers of prenatal alcohol exposure and FASD might exist and could have clinical utility [89]; importantly, these prenatal alcohol/FASD signatures were validated in a second cohort [90]. These findings set the stage for broader applications of DNA methylation in the context of FASD, creating a framework upon which to build future epigenomic studies of FASD and the development of biomarkers and assessment tools.

Chromatin modifications:

One step above DNA methylation lie the proteins – histones - that compact DNA in the nucleus [91]. Histones regulate access to the genetic material and gene expression patterns, which can be modulated through small chemical additions known as histone modifications. Importantly, these modifications play various roles depending on their genomic context and location on the histone and mediate the crosstalk between different levels of epigenetic regulation and cellular functions. In contrast to DNA methylation, the effects of alcohol on chromatin remain relatively understudied. Nevertheless, multiple lines of evidence suggest that developmental alcohol exposure can impact both the abundance and localization of different chromatin proteins and modifications. Several studies from animal models have shown differences in the levels of bulk histone modifications, generally revealing states less permissive to gene expression following prenatal alcohol exposure (*i.e.* genes turned off) (reviewed in [84]). A more recent study in post-mortem brains of individuals with FASD showed similar patterns of repressive chromatin profiles when assaying bulk levels across a range of brain regions and ages [92]. In addition to bulk approaches, gene-specific and genome-wide histone modification profiles have also been characterized in cell culture and animal models, with widespread effects being identified across different models and timing of exposures. As a whole, these results suggest that chromatin alterations are sensitive to the effect of alcohol and may play an important role in mediating the timing and dose-dependent effects of alcohol during development. In addition to histone modifications, several chromatin-associated proteins, such as MeCP2, can also regulate access to genes and modulate transcription factor recruitment to activate or repress gene expression. In particular, alterations to MeCP2 expression and regulation have been identified following alcohol exposure, suggestive of even broader alterations to chromatin profiles than suggested by altered histone modifications. It is also worth noting that mutations in MeCP2 lead to a severe neurodevelopmental disorder, Rett syndrome, suggesting that changes in its expression profiles could mediate some of the effects of alcohol [93].

Non-coding RNA:

The final layer of epigenetic regulation is mediated through non-coding RNA (ncRNA), which performs a wide variety of regulatory functions in the cell. These transcripts play important roles in the regulation of messenger RNA and protein levels and are central to the integration of physiological and molecular cues necessary for brain development [94]. The atypical expression of many ncRNA in the brain has also been associated with various neurological disorders, including autism spectrum disorder, Fragile X, Rett syndrome, schizophrenia, and anxiety-like disorder [95]. The vast majority of research on the effects of

alcohol on ncRNA has focused almost exclusively on micro RNA (miRNA), particularly with regards to alcohol-induced neurodegeneration and potential biomarkers of FASD (reviewed in [84]). Overall, these studies identified differentially expressed miRNAs with important roles in the development of deficits associated with prenatal alcohol exposure, including facial dysmorphisms, neuro-apoptosis, and altered neurodevelopment. Of note, the best characterized and replicated miRNA is miR-9, which displays altered expression patterns across multiple models and ages of alcohol exposure. Given its role in brain development and adult neural function, it may play an integral part in mediating some of the long-term deficits in FASD. Several miRNA have also been associated with prenatal alcohol later in adulthood, suggesting that they may retain a lasting signature of alcohol exposure and potentially act as biomarkers. A recent study followed up on this approach, showing that a panel of miRNA from the plasma of pregnant women who had consumed alcohol could predict infant neurodevelopmental outcomes on the Bayley scales of infant development at 6 and 12 months [96]. Although preliminary, these findings suggest that ncRNA could act as viable biomarkers of alcohol exposure, while also pointing to the biological underlying pathways influenced by alcohol; these latter findings could have important implications for the development of novel therapeutic interventions in FASD.

Considerations for epigenetic research on FASD÷

It is important to note that the vast majority of studies on epigenetic mechanisms have been performed in animal models and primarily in samples from male subjects, highlighting the need for sex-specific investigations of prenatal alcohol effects as well as replication of these findings using clinical cohorts of individuals with FASD. Furthermore, given that epigenetic modifications are sensitive to environmental effects, it is crucial to replicate these findings in additional studies and take into consideration other risk and resilience factors such as SEP, nutrition, and genetic background when identifying new biomarkers. Several epigenome-wide analyses have shown associations of many of these factors with DNA methylation patterns, which could potentially overlap with those identified in FASD, given that SEP, nutrition, and genetics can influence the manifestation of FASD. Finally, because epigenetics essentially provides an interface between genes and environment, changes in epigenetic marks will not only capture those that are biomarkers of prenatal alcohol exposure, but also those that are biomarkers of the many other social, economic, environmental, and nutritional risk factors for FASD. This will allow for new research directions that examine the social epigenetics underlying FASD outcomes. Future studies will likely take these into account to create more robust associations and biomarkers of prenatal alcohol exposure (Fig. 2).

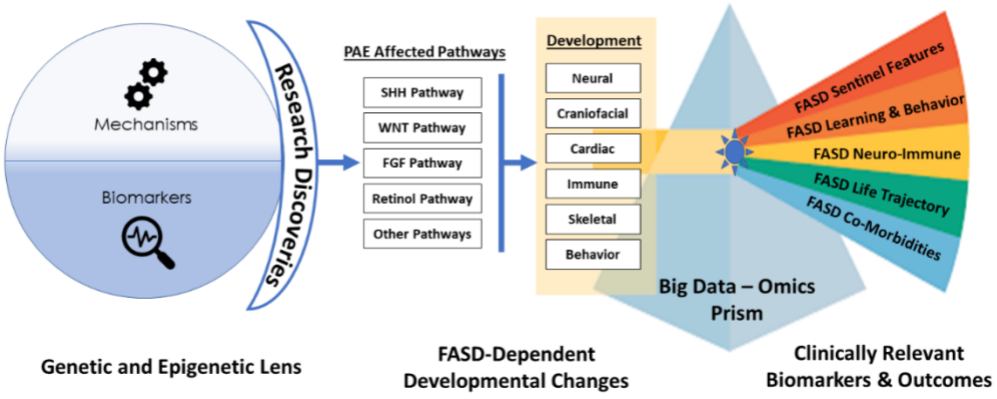

Figure 2: Schematic of genetic and developmental pathways and clinically relevant outcomes in FASD. Genetics and epigenetics play a large role in susceptibility and resilience in individuals prenatally exposed to alcohol. Several genetic pathways appear to be involved in the downstream effects of prenatal alcohol exposure (PAE) on development. The resulting FASD-relevant impairments to neural, craniofacial, cardiac, immune, skeletal, and behavioral systems lead to a deeper understanding of the molecular mechanisms that drive FASD-dependent developmental changes. Moreover, integration and analysis of large new "omics" data sets hold much promise to identify clinically relevant biomarkers and outcomes from infancy into adulthood that can provide a basis for early intervention and perhaps for discrimination among FASD outcomes, life trajectories, and FASD co-morbidities.

Section 5. Transcriptomic and proteomic alterations of FASD

Transcriptomic alterations:

Although epigenetic factors are an attractive potential mechanism to mediate the biological embedding of early life events, their association with transcription makes gene expressions programs a powerful target for assessing the molecular underpinning of alcohol-induced deficits. In particular, genome-wide investigations of gene expression programs have identified widespread alterations to gene expression levels in fetal, neonatal, and adult rodent models, providing important insight into potential mechanisms and pathways involved in alcohol-induced deficits. Given the importance of spatiotemporal gene expression during developmental patterning, it is perhaps not surprising that many of the alcohol-induced alterations to the transcriptome are closely related to the stage of development that was assessed [97]. For example, genes differentially expressed during gestation were generally associated with functions related to cellular patterning, growth, and development, suggesting that alcohol can interfere with typical developmental programs. As gene expression is highly dynamic, quickly responding to environmental and cellular inputs, transcriptional alterations measured soon after alcohol exposure may reflect the

intracellular response to the teratogen, rather than stable programming effects of prenatal alcohol on the genome. By contrast, gene expression profiling in the adult brain, long after the removal of alcohol, may provide additional insight into the long-term effects of prenatal alcohol exposure on cellular programs. Although these latter effects are usually subtler, long-lasting changes to the transcriptome have been identified in the brains of adult rodents, suggesting that alcohol can have lasting effects on the neural transcriptome [98,99].

By contrast, alterations identified in the entire embryo or brain likely reflect systemic effects of alcohol on the organism or CNS, respectively, and may reflect the broader effects of alcohol on biological functions. In particular, meta-analyses of gene expression patterns across multiple studies, ranging from whole embryos on embryonic day 9 in mice to the rat hippocampus on postnatal day 100, identified a general inhibition of transcription by alcohol, regardless of the model [97]. The differentially expressed genes identified in the combined analyses were mainly involved in protein synthesis, mRNA splicing, and chromatin function, suggesting that alcohol may broadly influence the regulatory systems of the cell, irrespective of the timing and dosage of alcohol exposure. More recent studies are beginning to focus on specific brain regions, providing insight into some of the functional deficits observed following prenatal alcohol exposure. For instance, gene expression patterns in the adult (postnatal day 70) mouse hippocampus are altered by a third trimester equivalent (~ postnatal days 1-10, the period of the brain growth spurt in rodents) exposure to binge levels of alcohol, which may potentially be related to some of the spatial learning and memory impairment associated with alcohol [100]. A recent study also profiled gene expression patterns in human fetal cortical tissue from late first trimester fetuses exposed to alcohol [101]. These embryos displayed a shift in the typical balance of splicing isoforms in addition to widespread alterations to transcriptomic programs, suggesting that alcohol may influence the fine balance of splice variants in the brain. However, these findings must be interpreted cautiously due to the small sample size (n=2). Nevertheless, taken together, these findings support the suggestion that gestational alcohol exposure can have both transient and persistent effects on the genome, which may influence the cellular response to alcohol and mediate the long-term deficits associated with FASD. Furthermore, alcohol-induced deficits may potentially arise through the disruption of epigenetic programs, concurrent with alterations to gene expression patterns.

Proteomic Alterations

Despite the valuable information gained from transcriptomic studies on the identity and level of gene expression altered by alcohol, changes in gene expression are often not directly correlated with changes in the amount of protein translated. While proteomic studies have generally been used to assess possible biomarkers of prenatal alcohol exposure in maternal-fetal interaction, including amniotic fluid and the uterine environment [102,103], there is also interest in studying proteomics in mammalian animal models during early development to elucidate the molecular pathways affected by prenatal alcohol exposure [104].

Treatment of dams with a high dose of alcohol on gestation day 8, mimicking a binge exposure, resulted in reduced amniotic fluid levels of alpha fetoprotein (AFP) compared to levels in control dams [104], suggesting that AFP could potentially be used as a biomarker for alcohol exposure. Importantly, this proteomic analysis of amniotic fluid on gestation day 17 (one day prior to birth) could discriminate between alcohol-exposed and unexposed embryos for high-risk of dysmorphogenesis [104]. Similarly, proteomic analysis of placentas of rats exposed to alcohol from gestation day 5-19 (mimicking a more chronic exposure) identified 45 significantly altered placental proteins between alcohol-exposed and control placentas: proteins involved in alcohol metabolism (alcohol dehydrogenases and aldehyde dehydrogenases), as well as genes involved in cellular function, immune function, nutrition, fetal and neurodevelopment, and implantation were upregulated in the alcohol-exposed cohort compared to pair-fed controls [102]. Finally, human placenta proteomic profiles obtained from alcohol-exposed and non-exposed pregnancies demonstrated significant reductions in placental expression of VEGFR2 and annexin-A4, both of which could serve as potential placental biomarkers for prenatal alcohol exposure [103]. Thus, proteomic profiling is a tool which could be used to identify novel biomarkers of FASD, while providing insight into its possible molecular underpinnings.

Section 6. Applications to Clinical Setting and Conclusions

FASD is the most common cause of neurodevelopmental impairments in the western world, with an estimated prevalence as high as 1.1-5%, affecting as many as 700,000 Canadians [11]. While prenatal alcohol exposure is the cause of FASD, not all alcohol-exposed individuals develop obvious deficits associated with FASD. In addition, determining if prenatal alcohol exposure has occurred is often very difficult due to the often subjective nature of self-reports of alcohol consumption or unavailability of the birth mother when assessing the child. Yet systematic reviews and meta-analyses have indicated that the global prevalence of alcohol use during pregnancy is 9.8% and it is estimated that 1 in 67 women who consume alcohol during pregnancy will deliver a child with FASD [105]. As we begin to understand the

growing complexity of prenatal alcohol exposure and the co-morbidity of risk factors like nutrition, socioeconomic position and stress – and all of these in the context of an individual's own genetic variation and epigenetic responses – it is clear that there is no known safe level of alcohol consumption that can be ascertained or generalized from the data available to date. Furthermore, given that one alcohol-sensitive developmental window occurs before a woman may know she is pregnant and that over half of the pregnancies in North America are unplanned, even the most important educational warning of "Don't drink if you are pregnant" will not be sufficiently effective. Innovative and integrative public education approaches are needed to allow couples in their reproductive years to understand better and more fully the risk between alcohol consumption during pregnancy and possible FASD outcomes.

New diagnostic tools that can provide sensitive biomarkers of prenatal alcohol exposure and that can be ethically applied would make a significant impact in the diagnosis and prevention of FASD. The current diagnostic process for FASD is comprehensive but extremely time-consuming and costly, requiring a team of medical, psychological, educational and social specialists. Note, however, that instruments are currently being developed that may help to expedite identification of individuals who might have been exposed to alcohol prenatally [106]. This could increase the ability to identify those who might be at risk for FASD, and these individuals could then be referred for the full team-based assessment, which would expedite diagnosis. Although some children with FAS can be identified in infancy, most FASD diagnoses occur much later once children are in school or even into adolescence and adulthood, and a large number of children on the spectrum may never receive a diagnosis, particularly if they don't have the dysmorphic facial features of FAS [18]. That said, it is well established that early cognitive, educational, adaptive, and behavioral interventions can profoundly change the long-term outcomes and quality of life of these individuals and their families [8,10]. Knowing this impact, early screening tools are essential to help identify at-risk children at a young age and provide an objective clinical assessment that will allow these children access to early interventions. Ensuring access to care for all children with FASD is a legislated right in Manitoba, as embodied by Jordan's Principle. Interestingly, the new ND-PAE diagnostic term ("neurobehavioral disorder associated with prenatal alcohol exposure") in the recent Diagnostic and Statistical Manual, 5th edition may be the exact key that facilitates early clinical evaluation with a recognized diagnostic assessment that allows access to early interventions. New medical guidelines and education programs are needed to incorporate such changes into a new standard health policy. The health care and education systems would need to be considered together under a broader framework to make this work. Importantly, we must also work to de-stigmatize FASD, identifying it as a neurodevelopmental

disorder rather than blaming or shaming the mother, thus removing the judgement and stigma that often surround mental health and addiction issues.

Identifying inexpensive and reliable biomarker(s) for prenatal alcohol exposure and associated FASD outcomes will lead to more accurate and earlier identification of those at greatest risk, particularly the vast majority of children with FASD who are currently invisible to FASD diagnosis. The presence of meconium fatty acid ethyl esters (FAEE) in newborn infants has been shown to highly correlate with alcohol exposure [107]. However, meconium FAEE can only capture heavy drinking in late pregnancy, which is often already known in the person's history, and may have little predictive value for FASD outcomes. The present review highlights the emerging potential for genetic, epigenetic, transcriptomic and proteomic approaches not only to elucidate FASD etiology, but also to serve as potential biomarkers or signatures of early-life events, including prenatal alcohol exposure.

In this age of "big data" and larger child and adult cohorts, new frameworks on how to integrate genetic and epigenetic data with those of the many recent omics studies will not only identify new clinically relevant biomarkers of FASD, but will help elucidate the underlying molecular etiology of FASD and the many FASD comorbidities and related neurodevelopmental disorders, as well as the molecular etiologies that clinically distinguish them. New frameworks will also point the way to potential new treatments/interventions for children with FASD and other neurodevelopmental disorders.

Bibliography:

1. Lemoine, P., Harousseau, H., Borteyru, J. & Menuet, J. Les enfants des parents alcoholiques: anomalies observées a propos de 127 cas. *Ouest Médical* **8**, 476–482 (1968).
2. Jones, K. L., DW, S., Ulleland, C. N. & Streissguth, A. P. Pattern of malformation in offspring of chronic alcoholic women. *Lancet* **1**, 1267–1271 (1973).
3. Streissguth A P *et al.* Natural history of the fetal alcohol syndrome: a 10-year follow-up of eleven patients. *Lancet* **2**, 85–91 (1985).
4. Norman, A. L., Crocker, N., Mattson, S. N. & Riley, E. P. *Neuroimaging and fetal alcohol spectrum disorders*. *Developmental Disabilities Research Reviews* **15**, 209–217 (2009).
5. Crocker, N., Nguyen, T. T. & Mattson, S. N. Fetal Alcohol Spectrum Disorders: Neuropsychological and Behavioral Features. **21**, 81–101 (2011).
6. Floyd, R. Fetal Alcohol Syndrome: Guidelines for Referral and Diagnosis. (2005).
7. Benz, J., Rasmussen, C. & Andrew, G. Diagnosing fetal alcohol spectrum disorder: History, challenges and future directions. *Paediatr. Child Health* **14**, 231–237 (2009).
8. Fox, S. E., Levitt, P. & Nelson III, C. A. How the Timing and Quality of Early Experiences Influence the Development of Brain Architecture. *Child Dev.* **81**, 28–40 (2010).
9. Paley, B. & O'Connor, M. J. Behavioral Interventions for Children and Adolescents With Fetal Alcohol Spectrum Disorders. *Alcohol Res. Heal.* **34**, 64–75 (2011).
10. Streissguth, A. P. *et al.* Risk factors for adverse life outcomes in fetal alcohol syndrome and fetal alcohol effects. *J. Dev. Behav. Pediatr.* **25**, 228–38 (2004).
11. May, P. A. *et al.* Prevalence of Fetal Alcohol Spectrum Disorders in 4 US Communities. *JAMA* **319**, 474–482 (2018).
12. Xu, G., Strathearn, L., Liu, B. & Bao, W. Prevalence of Autism Spectrum Disorder Among US Children and Adolescents, 2014-2016Prevalence of Autism Spectrum Disorder Among US Children and Adolescents, 2014-2016Letters. *JAMA* **319**, 81–82 (2018).
13. de Graaf, G., Buckley, F., Dever, J. & Skotko, B. G. Estimation of live birth and population prevalence of Down syndrome in nine U.S. states. *Am. J. Med. Genet. Part A* **173**, 2710–2719 (2017).
14. Clarren, S. K. & Smith, D. W. The Fetal Alcohol Syndrome. *N. Engl. J. Med.* **298**, 1063–1067 (1978).
15. Stratton, K., Howe, C. & Battaglia, F. *Fetal alcohol syndrome: Diagnosis, epidemiology,*

prevention and treatment. (National Academy Press, 1996).

16. Astley, S. J. & Clarren, S. K. Diagnosing the full spectrum of fetal alcohol-exposed individuals: introducing the 4-digit diagnostic code. *Alcohol Alcohol.* **35**, 400–410 (2000).
17. Hoyme, H. E. *et al.* Updated Clinical Guidelines for Diagnosing Fetal Alcohol Spectrum Disorders. *Pediatrics* **138**, (2016).
18. Chudley, A. E. *et al.* Fetal alcohol spectrum disorder: Canadian guidelines for diagnosis. *Can. Med. Assoc. J.* **172**, S1–S21 (2005).
19. Cook, J. L. *et al.* Fetal alcohol spectrum disorder: a guideline for diagnosis across the lifespan. *CMAJ* **188**, 191–197 (2016).
20. Kable, J. A. *et al.* Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure (ND-PAE): Proposed DSM-5 Diagnosis. *Child Psychiatry Hum. Dev.* **47**, 335–346 (2016).
21. Pierce, D. R. & West, J. R. Blood alcohol concentration: a critical factor for producing fetal alcohol effects. *Alcohol* **3**, 269–272 (1986).
22. Pierce, D. R., Goodlett, C. R. & West, J. R. Differential neuronal loss following early postnatal alcohol exposure. *Teratology* **40**, (1989).
23. Chernoff, G. F. The fetal alcohol syndrome in mice: An animal model. *Teratology* **15**, 223–229 (1977).
24. Randall, C. L. & Taylor, W. J. Prenatal ethanol exposure in mice: Teratogenic effects. *Teratology* **19**, 305–311 (1979).
25. Montag, A. C. Fetal alcohol-spectrum disorders: identifying at-risk mothers. *Int. J. Womens. Health* **8**, 311–323 (2016).
26. May, P. A. & Gossage, J. P. Maternal risk factors for fetal alcohol spectrum disorders: not as simple as it might seem. *Alcohol Res. Health* **34**, 15–26 (2011).
27. Abel, E. L. & Hannigan, J. H. Maternal risk factors in fetal alcohol syndrome: Provocative and permissive influences. *Neurotoxicol. Teratol.* **17**, 445–462 (1995).
28. Coles, C. Critical periods for prenatal alcohol exposure. *Alcohol health and research world* **18**, 22–29 (1994).
29. Dobbing, J. & Sands, J. Comparative aspects of the brain growth spurt. *Early Hum. Dev.* **3**, 79–83 (1979).
30. Guerri, C. Neuroanatomical and Neurophysiological Mechanisms Involved in Central Nervous System Dysfunctions Induced by Prenatal Alcohol Exposure. *Alcohol. Clin. Exp. Res.* **22**, 304–312 (1998).

31. Cudd, T. A. & Cudd, T. A. Animal model systems for the study of alcohol teratology. *Exp. Biol. Med.* **230**, 389–393 (2005).
32. May, P. A. *et al.* Maternal risk factors for fetal alcohol syndrome in the Western cape province of South Africa: a population-based study. *Am. J. Public Health* **95**, 1190–1199 (2005).
33. Weinberg, J. Nutritional issues in perinatal alcohol exposure. *Neurobehavioral toxicology and teratology* **6**, 261–269
34. Young, J. K., Giesbrecht, H. E., Eskin, M. N., Aliani, M. & Suh, M. Nutrition Implications for Fetal Alcohol Spectrum Disorder. *Adv. Nutr.* **5**, 675–692 (2014).
35. May, P. A. *et al.* Maternal nutritional status as a contributing factor for the risk of fetal alcohol spectrum disorders. *Reprod. Toxicol.* (2016). doi:10.1016/j.reprotox.2015.11.006
36. Krieger, N., Williams, D. R. & Moss, N. E. Measuring Social Class in US Public Health Research: Concepts, Methodologies, and Guidelines. *Annu. Rev. Public Health* **18**, 341–378 (1997).
37. Bingol, N. *et al.* The influence of socioeconomic factors on the occurrence of fetal alcohol syndrome. *Adv. Alcohol Subst. Abus.* (1987). doi:10.1300/J251v06n04_08
38. Lewis, P. T., Shipman, V. C. & May, P. A. Socioeconomic status, psychological distress, and other maternal risk factors for fetal alcohol spectrum disorders among American Indians of the Northern Plains. *Am. Indian Alaska Nativ. Ment. Heal. Res.* (2011). doi:10.5820/aian.1702.2011.1
39. Chrousos, G. P. & Gold, P. W. The Concepts of Stress and Stress System Disorders: Overview of Physical and Behavioral Homeostasis. *JAMA* **267**, 1244–1252 (1992).
40. Ruiz, R. J. & Avant, K. C. Effects of maternal prenatal stress on infant outcomes: A synthesis of the literature. *Advances in Nursing Science* (2005). doi:10.1097/00012272-200510000-00006
41. Coussons-Read, M. E., Okun, M. L., Schmitt, M. P. & Giese, S. Prenatal stress alters cytokine levels in a manner that may endanger human pregnancy. *Psychosom. Med.* (2005). doi:10.1097/01.psy.0000170331.74960.ad
42. McLachlan, K. *et al.* Dysregulation of the cortisol diurnal rhythm following prenatal alcohol exposure and early life adversity. *Alcohol* **53**, 9–18 (2016).
43. Yumoto, C., Jacobson, S. W. & Jacobson, J. L. Fetal substance exposure and cumulative environmental risk in an African American cohort. *Child Dev.* (2008). doi:10.1111/j.1467-8624.2008.01224.x
44. Abel, E. Paternal contribution to fetal alcohol syndrome. *Addict Biol* **9**, 126–127 (2004).

45. Gearing, R., McNeill, T. & A Lozier, F. Father involvement and fetal alcohol spectrum disorder: Developing best practices. *J FAS Int* **3**, (2005).
46. Day, J., Savani, S., Krempley, B. D., Nguyen, M. & Kitlinska, J. B. Influence of paternal preconception exposures on their offspring: through epigenetics to phenotype. *Am. J. Stem Cells* **5**, 11–18 (2016).
47. Hemingway, S. J. A. *et al.* Twin study confirms virtually identical prenatal alcohol exposures can lead to markedly different fetal alcohol spectrum disorder outcomes- fetal genetics influences fetal vulnerability. *Adv. Pediatr. Res.* **5**, 1–19 (2019).
48. Dworkin, S., Boglev, Y., Owens, H. & Goldie, S. The Role of Sonic Hedgehog in Craniofacial Patterning, Morphogenesis and Cranial Neural Crest Survival. *J. Dev. Biol.* **4**, 24 (2016).
49. Hong, M. & Krauss, R. S. Cdon mutation and fetal ethanol exposure synergize to produce midline signaling defects and holoprosencephaly spectrum disorders in mice. *PLoS Genet.* **8**, e1002999 (2012).
50. Ahlgren, S. C., Thakur, V. & Bronner-Fraser, M. Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 10476–81 (2002).
51. Kietzman, H. W., Everson, J. L., Sulik, K. K. & Lipinski, R. J. The teratogenic effects of prenatal ethanol exposure are exacerbated by sonic Hedgehog or Gli2 haploinsufficiency in the mouse. *PLoS One* **9**, 1–5 (2014).
52. Aoto, K., Shikata, Y., Higashiyama, D., Shiota, K. & Motoyama, J. Fetal Ethanol Exposure Activates Protein Kinase A and Impairs Shh Expression in Prechordal Mesendoderm Cells in the Pathogenesis of Holoprosencephaly. **231**, 224–231 (2008).
53. Gurdon, J. B. & Bourillot, P.-Y. Morphogen gradient interpretation. *Nature* **413**, 797–803 (2001).
54. Abramyan, J. Hedgehog Signaling and Embryonic Craniofacial Disorders. *J. Dev. Biol.* **7**, 9 (2019).
55. Huang, P. *et al.* Cellular Cholesterol Directly Activates Smoothened in Hedgehog Signaling. *Cell* **166**, 1176-1187.e14 (2016).
56. Logan, C. Y. & Nusse, R. THE WNT SIGNALING PATHWAY IN DEVELOPMENT AND DISEASE. *Annu. Rev. Cell Dev. Biol.* **20**, 781–810 (2004).
57. Lovely, C. Ben, Fernandes, Y. & Eberhart, J. K. Fishing for Fetal Alcohol Spectrum Disorders: Zebrafish as a Model for Ethanol Teratogenesis. *Zebrafish* **13**, 391–398 (2016).

58. Garic, A., Berres, M. E. & Smith, S. M. High-throughput transcriptome sequencing identifies candidate genetic modifiers of vulnerability to fetal alcohol spectrum disorders. *Alcohol. Clin. Exp. Res.* **38**, 1874–1882 (2014).
59. Lauing, K. L., Roper, P. M., Nauer, R. K. & Callaci, J. J. Acute Alcohol Exposure Impairs Fracture Healing and Deregulates b -Catenin Signaling in the Fracture Callus. *Alcohol. Clin. Exp. Res.* **36**, 2095–2103 (2012).
60. Reynolds, K., Kumari, P., Rincon, L. S., Gu, R. & Ji, Y. Wnt signaling in orofacial clefts : crosstalk , pathogenesis and models. *Dis. Model. Mech.* **12**, dmm037051 (2019).
61. Teven, C. M., Farina, E. M., Rivas, J. & Reid, R. R. Fibroblast growth factor (FGF) signaling in development and skeletal diseases. *Genes Dis.* **1**, 199–213 (2014).
62. Chrisman, K. *et al.* Gestational Ethanol Exposure Disrupts the Expression of FGF8 and Sonic hedgehog During Limb Patterning. **171**, 163–171 (2004).
63. Diez, R. & Storey, K. G. Opposing FGF and retinoid pathways : a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. *BioEssays* **26**, 857–869 (2004).
64. Wang, R. N. *et al.* Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis.* **1**, 87–105 (2014).
65. Serrano, M., Han, M., Brinez, P. & Linask, K. K. Fetal alcohol syndrome: cardiac birth defects in mice and prevention with folate. *Am. J. Obstet. Gynecol.* **203**, 75.e7-75.e15 (2010).
66. Burd, L. *et al.* Congenital Heart Defects and Fetal Alcohol Spectrum Disorders. *Congenit Hear. Dis.* **2**, 250–255 (2007).
67. Yelin, R. *et al.* Ethanol exposure affects gene expression in the embryonic organizer and reduces retinoic acid levels. *Dev. Biol.* **279**, 193–204 (2005).
68. Yelin, R., Kot, H., Yelin, D. & Fainsod, A. Early molecular effects of ethanol during vertebrate embryogenesis. *Differentiation* **75**, 393–403 (2007).
69. Johnson, C. S., Zucker, R. M., Sidney, E., Iii, H. & Sulik, K. K. Perturbation of Retinoic Acid (RA) -Mediated Limb Development Suggests a Role for Diminished RA Signaling in the Teratogenesis of Ethanol. *Birth Defects Res. (Part A)* **641**, 631–641 (2007).
70. Eriksson, C. J. The role of acetaldehyde in the actions of alcohol. *Alcohol Clin Exp Res.* **25**, 15S--32S (2001).
71. Jacobson, S. W. *et al.* Protective effects of the alcohol dehydrogenase-ADH1B allele in children exposed to alcohol during pregnancy. *J Pediatr.* **148**, 30–37 (2006).

72. Driscoll, D. A. *et al.* Prevalence of 22q11 microdeletions in DiGeorge and velocardiofacial syndromes: implications for genetic counselling and prenatal diagnosis. *J. Med. Genet.* **30**, 813–817 (1993).
73. Cook, J. L. *et al.* Fetal alcohol spectrum disorder: a guideline for diagnosis across the lifespan. *CMAJ* 1–7 (2016). doi:10.1503/cmaj.141593/-/DC1
74. Lammer, E. J. *et al.* Retinoic Acid Embryopathy. *N. Engl. J. Med.* **313**, 837–841 (1985).
75. Sanlaville, D. *et al.* Phenotypic spectrum of CHARGE syndrome in fetuses with CHD7 truncating mutations correlates with expression during human development. *J. Med. Genet.* **43**, 211–217 (2006).
76. Legendre, M. *et al.* Phenotype and genotype analysis of a French cohort of 119 patients with CHARGE syndrome. *Am. J. Med. Genet.* **175C**, 417–430 (2017).
77. Hartshorne, T. S., Grialou, T. L. & Parker, K. R. Autistic-Like Behavior in CHARGE Syndrome. *Am. J. Med. Genet.* **133A**, 257–261 (2005).
78. Bakker, G. *et al.* Distinct white-matter aberrations in 22q11.2 deletion syndrome and patients at ultra-high risk for psychosis. *Psychol. Med.* **46**, 2299–2311 (2016).
79. Campbell, L. E. *et al.* Brain and behaviour in children with 22q11.2 deletion syndrome: A volumetric and voxel-based morphometry MRI study. *Brain* **129**, 1218–1228 (2006).
80. Sulik, K. K., Cook, C. S. & Webster, W. S. Teratogens and craniofacial malformations: Relationships to cell death. *Development* (1988).
81. Baldini, A. Dissecting contiguous gene defects: TBX1. *Curr. Opin. Genet. Dev.* **15**, 279–284 (2005).
82. Bird, A. Perceptions of epigenetics. *Nature* **447**, 396–398 (2007).
83. Ziller, M. J. *et al.* Charting a dynamic DNA methylation landscape of the human genome. *Nature* **500**, 477–481 (2014).
84. Lussier, A. A., Weinberg, J. & Kobor, M. S. Epigenetics studies of fetal alcohol spectrum disorder: where are we now? *Epigenomics* **9**, (2017).
85. Jones, P. A. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat. Rev. Genet.* **13**, 484–492 (2012).
86. Bock, C. Epigenetic biomarker development. *Epigenomics* **1**, 99–110 (2009).
87. Liu, Y., Balaraman, Y., Wang, G., Nephew, K. P. & Zhou, F. C. Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation. *Epigenetics* **4**, 500–11 (2009).
88. Lussier, A. A. *et al.* Prenatal Alcohol Exposure: Profiling Developmental DNA Methylation

Patterns in Central and Peripheral Tissues. *Front. Genet.* **9**, 610 (2018).

89. Portales-Casamar, E. *et al.* DNA methylation signature of human fetal alcohol spectrum disorder. *Epigenetics Chromatin* **9**, 81–101 (2016).
90. Lussier, A. A. *et al.* DNA methylation as a predictor of fetal alcohol spectrum disorder. *Clin. Epigenetics* **10**, 5 (2018).
91. Kouzarides, T. Chromatin Modifications and Their Function. *Cell* **128**, 693–705 (2007).
92. Jarmasz, J. S. *et al.* Global DNA Methylation and Histone Posttranslational Modifications in Human and Nonhuman Primate Brain in Association with Prenatal Alcohol Exposure. *Alcohol. Clin. Exp. Res.* **43**, 1145–1162 (2019).
93. Bogdanović, O. & Veenstra, G. J. C. DNA methylation and methyl-CpG binding proteins: Developmental requirements and function. *Chromosoma* **118**, 549–565 (2009).
94. Wang, K. C. & Chang, H. Y. Molecular Mechanisms of Long Noncoding RNAs. *Molecular Cell* **43**, 904–914 (2011).
95. Vieira, A. S., Dogini, D. B. & Lopes-Cendes, I. Role of non-coding RNAs in non-aging-related neurological disorders. *Brazilian J. Med. Biol. Res. = Rev. Bras. Pesqui. medicas e Biol.* **51**, e7566–e7566 (2018).
96. Balaraman, S. *et al.* Plasma miRNA Profiles in Pregnant Women Predict Infant Outcomes following Prenatal Alcohol Exposure. *PLoS One* **11**, e0165081 (2016).
97. Rogic, S., Wong, A. & Pavlidis, P. Meta-analysis of gene expression patterns in animal models of prenatal alcohol exposure suggests role for protein synthesis inhibition and chromatin remodeling. *Alcohol. Clin. Exp. Res.* **40**, 717–727 (2016).
98. Kleiber, M. L., Mantha, K., Stringer, R. L. & Singh, S. M. Neurodevelopmental alcohol exposure elicits long-term changes to gene expression that alter distinct molecular pathways dependent on timing of exposure. *J. Neurodev. Disord.* **5**, 6 (2013).
99. Lussier, A. A. *et al.* Prenatal Alcohol Exposure Alters Steady-State and Activated Gene Expression in the Adult Rat Brain. *Alcohol. Clin. Exp. Res.* **39**, 251–261 (2015).
100. Chater-Diehl, E. J., Laufer, B. I., Castellani, C. A., Alberry, B. L. & Singh, S. M. Alteration of gene expression, DNA methylation, and histone methylation in free radical scavenging networks in adult mouse hippocampus following fetal alcohol exposure. *PLoS One* **11**, e0154836 (2016).
101. Kawasawa, Y. I. *et al.* Genome-wide profiling of differentially spliced mRNAs in human fetal cortical tissue exposed to alcohol. *Alcohol* **62**, 1–9 (2017).
102. Davis-Anderson, K. L. *et al.* Placental Proteomics Reveal Insights into Fetal Alcohol Spectrum

Disorders. *Alcohol. Clin. Exp. Res.* **41**, 1551–1558 (2017).

103. Holbrook, B. D. *et al.* The association between prenatal alcohol exposure and protein expression in human placenta. *Birth Defects Res.* 1–11 (2019). doi:10.1002/bdr2.1488
104. Datta, S. *et al.* Fetal Alcohol Syndrome (FAS) in C57BL / 6 Mice Detected through Proteomics Screening of the Amniotic Fluid. *Birth defects Res. Part A. Clin. Mol. Teratol.* **82**, 177–186 (2008).
105. Popova, S., Lange, S., Probst, C., Gmel, G. & Rehm, J. Global prevalence of alcohol use and binge drinking during pregnancy, and fetal alcohol spectrum disorder. *Biochemistry and Cell Biology* (2018). doi:10.1139/bcb-2017-0077
106. Goh, P. K. *et al.* A Decision Tree to Identify Children Affected by Prenatal Alcohol Exposure. *J. Pediatr.* **177**, 121-127.e1 (2016).
107. Ostrea, E. M. *et al.* Fatty Acid Ethyl Esters in Meconium: Are They Biomarkers of Fetal Alcohol Exposure and Effect? *Alcohol. Clin. Exp. Res.* **30**, 1152–1159 (2006).