# THE BRAIN AND BEYOND: MATERNAL AND FETAL TARGETS OF CHRONIC ALCOHOL EXPOSURE IN PREGNANCY

A Dissertation

by

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# ABSTRACT

Fetal alcohol spectrum disorders (FASD) persists as significant a public health threat partly due to enmeshment of drinking culture in modern society and partly because of the inherent complexity of alcohol-mediated pathogenesis. Discernment of FASD pathogenesis remains warranted because of the persistence of this threat coupled with extremely limited treatment options for affected patients. In the following studies (Chapters 2 & 3) we used classic approaches to describe a potential mechanism for alcohol-mediated pathogenesis in an atypical focal point of FASD investigation: the maternal uterine artery. The latter part of this work (Chapters 5 & 6) applied advanced technologies (HPLC, next-gen RNA sequencing) to identify new foci in a classic FASD target: the fetal brain. All studies were completed using a well characterized *in vivo* model of chronic binge prenatal alcohol exposure with clinical relevance. In the uterine artery, alcohol impaired the myogenic response and endothelial-mediated vasodilation, and dysregulation of the nitric oxide (NO) pathway and the enzyme responsible for NO synthesis (eNOS) were presented as susceptible candidates for this dysfunction. Brain studies described here bolster support for pursuing investigation of how excitatory amino acid imbalances influence neurotoxicity, expressly in the developing cerebellum and hippocampus. Transcriptome analysis also identified new hippocampal genes and canonical pathways of investigational interest based on their previous linkage to alcohol and FASD-adjacent pathology but were not tied to FASD contextually to FASD until now. Ultimately, we affirmed that alcohol use during pregnancy is unsafe and poses significant health risks not only to fetal development but to maternal physiology essential for sustaining this development.

# DEDICATION

This work is dedicated to myself and to Timothy Cudd, DVM, PhD. The second to last thing he ever told me was that he just wasn't done learning, and the document that follows is additional learning on his behalf. The last thing he told me was that I could be anyone I wanted to be or do anything I wanted to do without limitation.

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# NOMENCLATURE

| | |
|---|---|
| Ach | Acetylcholine |
| Alcohol | Ethyl alcohol ($C_2H_5OH$) |
| ARBD | Alcohol-Related Birth Defect(s) |
| ARND | Alcohol-Related Neurodevelopmental Disorder |
| AUD | Alcohol Use Disorder |
| BAC | Blood Alcohol Content |
| CNS | Central Nervous System |
| CVA | Cerebrovascular Accident |
| EDHF | endothelial-derived hyperpolarizing factor |
| eNOS | Endothelial Nitric Oxide Synthase |
| FAS | Fetal Alcohol Syndrome |
| GD | Gestational Day |
| IUGR | Intrauterine Growth Restriction |
| MRI | Magnetic Resonance Imaging |
| NO | Nitric Oxide |
| PAE | Prenatal Alcohol Exposure |
| PFAS | Partial Fetal Alcohol Syndrome |
| PND | Postnatal Day |
| PGI2 | Prostacyclin |
| SIDS | Sudden Infant Death Syndrome |
| Tbx | Thromboxane |

| | |
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| UBF | Uterine Blood Flow |

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

# 1. INTRODUCTION

## 1.1. FASD: A Brief History and Present Perspectives

Ethyl alcohol (alcohol) is a notorious teratogen, and its capacity for fetal harm has been described throughout history for millennia. Early allusions to this phenomenon precede the Common Era, appearing in the Old Testament and scholastic texts by the ancient Greeks and Romans. The first modern-format scientific literature describing its teratogenicity was published over a century ago in the 1890s by Scottish physician Charles Templeman. In a case study of 258 infants with presumed asphyxiation-related death [or likely, Sudden Infant Death Syndrome (SIDS)], Templeman listed maternal alcohol abuse as a major contributing factor to infant mortality[1,2]. Subsequent case studies by British and French physicians at the turn of the twentieth century identified various risks alcohol poses to pregnancy and perinatal health[3–6]. Despite these compelling accounts that directly correlated alcohol use in pregnancy with fetal detriment, the topic of alcohol's teratogenicity did not gain a firm foothold in modern literature until the late 1950s and 1960s, when two key French studies emerged[7,8].

Jaqueline Roquette, a doctoral student, was the first to link prenatal alcohol exposure (PAE) with low birth weight and behavioral abnormalities in children[7]. Roughly a decade after her, French physician Paul Lemoine associated PAE with craniofacial dysmorphology and developmental growth restriction that presented phenotypically in neonatal patients as low birthweight[8]. Low birthweight had previously been connected with increased risk for infant comorbidities and mortality[9,10], and the joint efforts by Roquette and Lemoine resonated with the global perinatal research community. Ultimately, these preliminary reports sparked a global movement to advance perinatal health outcomes and combat alcohol use during pregnancy[6].

Recognition remains limited for these initial case studies, but they forged a solid foundation for future works by an American pediatrician group at the University of Washington to launch from. These American studies were published several years later yet are widely viewed today as the preeminent works that defined alcohol's teratogenicity.

In 1973, Washington physicians Kenneth L. Jones, David W. Smith, Christy N. Ulleland, and Ann P. Streissguth published a case study in the *LANCET* that identified a novel pattern of birth defects in 8 children born to alcoholic mothers, which included craniofacial dysmorphology and cardiovascular aberrations linked previously to fetal growth restriction[11]. A noteworthy caveat of this study was that the patients assessed by Jones and colleagues were unrelated and represented three different ethnicities[11]. Later that year, Jones and Smith published a case study of three Native American patients all under the age of one, who were born to mothers with a known history of alcohol abuse[12]. It was here that Jones and Smith coined the term Fetal Alcohol Syndrome (FAS) to clinically describe the combined presentation and pathology of what is now considered to be the most severe health outcome resulting from fetal alcohol exposure[12]. Three specific phenotypic features in patients are requisite for an FAS diagnosis:

1) growth impairment,
2) craniofacial dysmorphology, and
3) central nervous system (CNS) dysfunction and/or learning/behavioral abnormalities[13,14].

These developmental damages were described as permanent and irreversible, with a profound impact on patient quality of life[11,12]. In response to these influential papers by Jones and colleagues, efforts to discern alcohol's teratogenicity gained traction and momentum on a global scale among researchers whose interests involved maternal, fetal, and pediatric health.

The collective investigative response to the foundational contributions from Roquette, Lemoine, Jones, Smith, Ulleland, and Streissguth resulted in a major recurring finding: PAE could lead to an array of developmental deficits that did not fully align with the diagnostic criteria established for FAS. The Institute of Medicine (IOM) was the first group to describe a wide-ranging spectrum of PAE-related health effects in pediatric patients and issued initial diagnostic criteria for this novel spectrum in 1996[15]. After a subsequent inquiry, this spectrum of health outcomes was further subdivided into the following categories:

1) FAS,
2) Partial FAS (PFAS),
3) Alcohol-Related Neurodevelopmental Disorder (ARND), and
4) Alcohol-Related Birth Defect(s) (ARBD).

In 2003, thirty years after Jones' and colleagues' initial FAS reports, a new term, Fetal Alcohol Spectrum Disorders (FASD), was introduced to more precisely describe a collection of all possible alcohol-mediated developmental outcomes[14].

Despite the known cause of FAS and other FASD subcategories, accurate clinical assessment of patients remains nebulous due to inherent challenges:

1) lack of a universal diagnostic standard,
2) symptom overlap with many other developmental disorders,
3) persistent knowledge gaps and misconceptions among primary healthcare providers, and
4) a lack of reporting alcohol use by mothers due to social stigma[15–17].

These challenges were a major factor why clinical diagnostic criteria for FAS alone were not standardized in the U.S. until 2004, following a 2002 congressional mandate (and perhaps the

Canadian contingent setting precedent[18]) as a joint effort by the CDC, National Center on Birth Defects and Developmental Disabilities (NCBDDD), and Department of Health and Human Services (DHS)[19]. To date, the CDC views FASD as a range of alcohol-mediated developmental disorders and not a diagnostic term. Thus, FASD specifically lacks government-issue diagnostic criteria, at least in the U.S (Canada, for instance uses FASD as a diagnostic term as of 2015)[19–21]. Guidelines for classifying the array of pathologies comprised within FASD were first described in 2005 by an international group of FASD specialists in *Pediatrics*, and this framework is continuously updated and refined in clinician databases (e.g., UpToDate®, Medline) as knowledge is gained through the literature about FASD pathology[16,17,20–23]. In brief, the current diagnostic criteria for FASD subcategories are comprised in Table 1[22].

**Table 1:** *FASD Subcategories and Diagnostic Criteria.* Patient history refers to whether prenatal alcohol exposure is known (+ PAE) or unknown/undocumented (- PAE).

| | Classification | Acronym | Patient History | Diagnostic Criteria |
|---|---|---|---|---|
| **Fetal Alcohol Spectrum Disorders (FASD)** | **Fetal Alcohol Syndrome** | **FAS** | **+ / - PAE** | ▪ Growth deficit(s)<br>▪ Impaired brain development<br>▪ Behavioral deficits<br>▪ Craniofacial dysmorphology |
| | **Partial Fetal Alcohol Syndrome** | **PFAS** | **+ PAE** | ▪ Behavioral deficits<br>▪ Craniofacial dysmorphology |
| | | | **- PAE** | ▪ Growth restriction, impaired brain development *or* impaired neurological function<br>▪ Behavioral deficits<br>▪ Craniofacial dysmorphology |
| | **Alcohol-Related Neurodevelopmental Disorder** | **ARND** | **+ PAE** | ▪ Neurological, learning, and/or behavioral deficits |
| | **Alcohol-Related Birth Defect(s)** | **ARBD** | **+ PAE** | ▪ Anatomic malformation(s) known to be caused by PAE |

FASD is popularly described in the literature as an 'umbrella term,' since it encompasses all possible health consequences resulting from PAE[14,20]. The developing brain is the most studied target, largely because the effects that alcohol has on the developing brain are patent, perceptible, and extensive in nature[24–28]. Similar to FAS, brain and related CNS deficits observed in FASD patients are lifelong with serious implications for patients' quality of life, although studies show early intervention is efficacious at improving outcomes in affected individuals[29–31]. Alcohol-mediated developmental brain damage may manifest as an array of:

1) cognitive and behavioral deficits [e.g., impaired verbal learning retention[32] and impaired inhibition, working memory, and executive function[33]];
2) structural abnormalities [e.g., diffusion abnormalities in both white and gray matter regions[34,35] and MRI-assessed functional connectivity abnormalities[25,36]];
3) brain region- or cell-specific changes [e.g., cerebellar Purkinje cell loss with lobule-specific changes resulting from differential exposure dose and timing[37–39]].

The wide-ranging effects alcohol exerts on the developing brain pose a fundamental challenge to the development of effective treatment and intervention strategies for patients with brain-related FASD pathologies[27,28,40].

Despite the exquisite vulnerability the fetal brain demonstrates in response to alcohol, studies show nearly every developing organ system may be vulnerable to *in utero* alcohol exposure, including the skeletal, cardiovascular, gastrointestinal, renal, hepatic, immune, and endocrine systems[41–45]. These developing systems and their respective organs likewise show varied susceptibility to alcohol, although not to the same degree as does the developing brain. This broad-spectrum vulnerability to PAE presents clinically as an array of heterogenic outcomes and is oppositional to discerning the underlying mechanisms – the molecular 'how' and 'why' –

that are responsible for alcohol-mediated damage. Alcohol's teratogenesis is further compounded by the fact that the degree to which alcohol impairs fetal development is dependent on exposure dose, timing, and duration[38,46–48].

A global investigative effort has extensively chronicled the developmental damage associated with FASD via human and translational animal model studies. For as many organ and tissue types that show teratogenic susceptibility to alcohol, a myriad of potential mechanisms has been proposed[49–57]. Validation for many of these mechanisms remains lacking. Another contributing factor to the obscurity of PAE-mediated pathology may be that the proposed theoretical multi-mechanistic actions of alcohol may have synergistic or additive effects, further compounding alcohol's capacity for developmental harm. In addition to its teratogenic properties, alcohol is also a toxicant, with profound physiological effects even at exceedingly low levels, further adding to its actions' complexity[58]. Thus, a pervasive lack of mechanistic understanding has impeded effective intervention approaches and treatment strategies for patients with FAS/FASD. Currently, no approved pharmaceutical treatment exists for treating any symptom or pathology specific to FASD patients[29,30].

The collective response to FASD recognition was more multidisciplinary relative to the FAS outset. One branch of pre-clinical research sought to elucidate the mechanisms underlying alcohol-mediated developmental damage. Other branches catalogued this damage, investigated potential therapeutics, and developed behavioral intervention strategies for mitigating the effects of alcohol-mediated damage incurred *in utero*. Epidemiologists assessed FASD prevalence among various populations world-wide and identified risk factors for likely to increase incidence. In concert with research, educators and social outreach campaigns cultivated a common public understanding for the material threat alcohol imparts to pregnancy. Public

awareness initiatives were launched nationally, particularly because at the time of Jones' and colleagues' preliminary FAS work, alcohol was clinically indicated as an accessible, cost-efficient tocolytic for blocking preterm labor, despite a profound lack of clinical evidence supporting its efficacy[59]. The FDA and CDC issued advisories to promote public awareness of the potential dangers alcohol poses to pregnancy, and the U.S. Surgeon General issued recurrent warnings stating that no amount of alcohol was considered safe during pregnancy[60–62]. This guideline urging pregnant women to abstain from alcohol was similarly endorsed by the American Academy of Pediatrics[23]. Based on the scope of these public educational platforms, it's equitable to question the demand for additional FASD research, given that the major hazards of drinking during pregnancy are largely known (growth restriction, craniofacial abnormalities, and neurological deficits), and it's also logical to reason whether FASD could be eradicated simply by abstaining from alcohol. In fact, numerous papers have attributed PAE as the leading cause of preventable mental neurobehavioral and developmental deficits[31,63–67]. The answer as to why this predicament remains relevant lies in the complex actions of the deceptively small ethanol molecule ($C_2H_5OH$).

### 1.2. Alcohol: America's Drug of Choice

America's relationship with alcohol is nearly as complicated as alcohol's actions are on the body, and because of its profundity, addressing this relationship is necessary for a holistic understanding of FASD. The FASD field is small but heterogenous, yet for reasons unknown, cross talk remains lacking between this alcohol-related field and those involving mental health, alcohol abuse, and addiction in the context of young adults of reproductive age. Commendable strides have improved our understanding of FASD epidemiology [e.g., its prevalence in specific

communities[68] and its penetrance via novel diagnostic biomarkers for detecting *in utero* alcohol exposure[69,70]]. However, singularly assessing this without also understanding the epidemiology of alcohol as a drug of abuse leaves fundamental questions unanswered. Adequate assessment of America's relationship with alcohol is imperative for recognizing the underlying forces oppositional to FASD prevention and treatment, such as why many individuals cannot simply abstain from drinking.

Alcohol is a psychoactive drug that is predominantly legalized worldwide and is ubiquitously used for recreational purposes on account of its actions as a central nervous system (CNS) depressant. It is a unique drug, in that it is a voluntarily consumed toxicant, often in unregulated amounts[58]. It is by far the most commonly used recreational drug by Americans, as evidenced by the 2018 National Survey on Drug Use and Health (NSDUH), which reported that in the past month, 139.8 million study participants drank alcohol, in contrast to the 58.8 million and 31.9 million participants who respectively used tobacco or illicit drugs[71]. Alcohol is effective, accessible, and economical, and these attributes justify its pervasive use. It is also imbued in Western culture as a social lubricant and as a means for celebration, relaxation, and revelry. In the U.S., the alcohol industry is a major economic driver, responsible for generating $363.33 billion in total annual revenue[72]. American alcohol sales generate a yearly tax revenue of $47.9 billion and the alcohol industry is credited with creating 2.03 million jobs[72]. Collectively, the alcohol industry comprises 1.65 percent of the American GDP[72]. U.S. consumption is estimated to be 2.35 gallons per capita, which roughly translates to 576 million gallons of alcohol consumed each year[72]. To say that Americans appreciate their spirits would be an understatement, and they vehemently defend their right to use this potent anxiolytic with as few limitations as permissible.

The crux of alcohol is that it poses a profound threat to the society that embraces it. Alcohol is an addictive, behavioral-altering drug, that accounts for an estimated 88,000 American deaths each year, making it the third most-preventable cause of death after tobacco and poor diet/lack of exercise[73,74]. In parallel with FASD, alcohol abuse in adults alone is implicated in a wide path of preventable destruction. Deaths attributed to alcohol abuse are subcategorized as having acute or chronic etiology, and the divergence of both categories is profound. *Chronic* causes of alcohol-mediated death include pancreatitis, esophageal cancer, gastroesophageal hemorrhage, hepatitis, nervous system degeneration, cirrhosis, cerebrovascular accident (CVA or stroke), cardiomyopathy, and ischemic heart disease, among copious other means[75]. *Acute* causes of alcohol-mediated death include asphyxiation, drowning, motor vehicle crashes, hypothermia, homicide, and alcohol poisoning[75]. In added congruence with FASD, the myriad of ways in which alcohol abuse in adults alone can lead to death shows how alcohol can impair or even result in failure of nearly every major organ system, particularly those that include vital organs, such as the heart, brain, and liver.

Adding to alcohol's complexity is that as much as it is an economic driver, alcohol is paradoxically an economic deterrent. Most recently assessed in 2010, the economic burden of excessive American drinking exceeded $249 billion, drastically narrowing alcohol's revenue margin[76]. Consequential costs included productivity losses, healthcare costs incurred for medical treatment, and law enforcement and related judicial expenses[76]. Thus, alcohol poses significant risks to not only individuals but also to society as a whole[77]. This raises an important question: if alcohol is so potentially harmful, how is its use so ubiquitous in society?

To address this question in the context of FASD, it is essential to understand the prevalence of alcohol use among adults of reproductive age, particularly among women who are

or may become pregnant. A recent study assessing the adult reproductive age cohort (18-44 years) indicated that over half of nonpregnant women report alcohol use in the past month (defined as 30 days)[78]. Since nearly half of pregnancies in the U.S. are unplanned, this accounts in part for how alcohol exposure during pregnancy persists[79–81]. Tan and colleagues also reported past month alcohol use among pregnant women: 1 in 10 reported drinking in 2015, and in their most recent report, this metric increased to 1 in 9[78,82]. It is important to note that despite roughly half a century of research and public education outreach efforts, incidence of FASD has not decreased[83]. The most recent reports estimate that FASD affects 2-5% of American school-age children, with certain populations having an FASD incidence as high as 9%[68,84–86]. Collectively, these consumption, prevalence, and incidence rates justify the significance of maternal alcohol use during pregnancy as a significant and persistent public health issue.

These substantial frequency rates of both maternal drinking and FASD invoke two pertinent questions:

1) Why is it that a beverage that is known to be harmful and even deadly is consumed in mass? and
2) Why do many pregnant women cease to abstain?

A central rationale for the first query is that alcohol is a habit forming and highly addictive drug, freely accessible to adults aged 21 and over nationwide, and is an incredibly potent anxiolytic and social catalyst, making it wildly popular. The reason why many drinkers including pregnant women do not abstain despite well-known health risks is that due to alcohol's addictive properties, many who imbibe in aberrant drinking behaviors are chemically dependent and/or physically unable to abstain.

Aberrant drinking behavior that results in distress or harm is medically classified by Diagnostic and Statistical Manual of Mental Disorders (DSM–5) as Alcohol Use Disorder (AUD). An AUD is defined by the National Institute for Alcohol and Alcoholism (NIAAA) as, "chronic relapsing brain disease characterized by compulsive alcohol use, loss of control over alcohol intake, and a negative emotional state when not using," and includes such behaviors as excessive drinking, binge drinking, increasing consumption to achieve the desired effect, difficulty or inability to control drinking, and alcoholism[87]. Treatment for AUDs is often inaccessible, costly, and ineffective. Since AUD treatment can be cost prohibitive, those without health insurance or financial means are less likely to seek or receive care[88]. In 2017, 14.8 million Americans were reported to have an AUD, yet only 4.2% received treatment[88]. In other words, the most recent national assessment estimates that 9 out of 10 Americans with an AUD did not receive treatment. Interestingly, AUD incidence was highest among individuals of reproductive age (12-44 years) when compared with older age demographics[88]. Relapse rates are alarmingly high among AUD patients, as roughly half will relapse within a year after completing treatment[89,90]. Therefore, alcohol's addictive properties coupled with the mental health crisis that accompanies those who abuse this drug have led to a major public health issue that has proven extremely difficult to effectively curtail.

For chemically dependent users, abrupt alcohol cessation can result in hallucinations, seizures, and *delirium tremens*, which can cause dangerous shifts in homeostatic regulation and can subsequently result in death[91,92]. Thus, despite extensive evidence of the threat alcohol poses to pregnancy, mothers who are addicted to alcohol cannot suddenly quit drinking without risking serious health consequences[79,82,93]. In summary, although alcohol is molecularly simple, the health, societal, and economic implications of this widely abused drug are far-reaching and

multifaceted, and this complexity has proven extremely difficult to account for in the context of treating and preventing FASD, which remains a significant public health threat.

### 1.3. Thunderstruck: Significance and Relevance of a Binge Model

Binge drinking is an aberrant drinking behavior defined in women as consuming 4 or more alcoholic beverages per drinking episode and poses an explicit threat to fetal development, as it is often associated with higher blood alcohol content (BAC)[94,95]. Since alcohol is a CNS depressant, the adult brain adapts to elevated BACs by increasing the excitability of neurons[96,97]. This compensatory shift explains how chronic heavy drinkers are able to appear coherent and/or functional with relatively high BACs, the same BACs that would render non-heavy drinkers incapacitated. Small, uncharged, lipophilic ethanol molecules easily cross into the fetal compartment via the placenta, and binge-associated BACs pose an even higher threat to the circuitry and cell populations in the developing brain than in the adult the mother[37,98]. Alcohol has been consistently described as having a dose dependent effect on FASD phenotypic severity, and the higher BACs associated with binge drinking is a major reason why this particular consumption pattern is profoundly harmful to fetal development[37,93,94,99,100].

The consumption pattern specific to binge drinking also increases potential for fetal harm. A chronic binge pattern entails heavy drinking followed by a period of withdrawal, repeating over time[82,101]. Withdrawal episodes can cause acute alcohol withdrawal syndrome which alone poses a risk to healthy pregnancy due to eliciting symptoms of headache, anxiety, dehydration, and autonomic instability[102,103]. Repeated withdrawal episodes pose an additional health threat: they exhibit a phenomenon called *kindling*. Kindling is when symptom severity increases with each consecutive episode[104]. Chronic binge drinking produces repeated

withdrawal episodes which induce more severe symptoms: tremors, seizures, and profound autonomic dysregulation[91,102,103]. Thus, any withdrawal symptoms in the mother that compromise fetal health may pose a greater fetal risk as frequency increases. It is well documented in human and animal model studies that cyclical BAC spiking and falling coupled with recurrent alcohol withdrawal episodes impair a myriad of brain functions, and the developing brain has shown exquisite vulnerability to binge exposure patterns[37–39,47,93,94,99,102,103,105–118]. Collectively, these characteristics of binge drinking explain why it is particularly harmful to fetal health.

Nearly 20% of reproductive-age women (18-44 years) report binge drinking within the past 30 days[78]. Adolescents and young adults comprising a portion of the reproductive age demographic are also more likely to engage in risky drinking behaviors, such as binge drinking[78,82,119,120]. Conversely, reproductive age women who binge drink run a higher likelihood of unintended pregnancy and are also more likely to continue drinking after conception[121,122]. Specifically among pregnant women, a 2015 study by Tan and colleagues reported that 1 in 33 binge drank in the past month[78]. Surprisingly, their most recent assessment of this demographic found this rate had increased to 1 in 26[82]. The latter 2019 study further assessed binge drinking parameters within its cohort of pregnant women: binge drinking frequency averaged 4.5 episodes per month and amount of alcohol consumed per episode averaged 6.0 drinks[82]. Based on the explicit risks binge drinking poses to pregnancy (i.e., unintentional heavy alcohol-exposure during the periconceptional period) and its frequency among potentially and reportedly pregnant women, a binge drinking paradigm is socially pertinent to model. Binge drinking is also a clinically relevant pattern to study because it correlates with more severe phenotypes on the FASD spectrum (e.g., FAS, PFAS), such as craniofacial dysmorphology and profound CNS deficits[93]. This exposure paradigm has been shown to reproduce analogous phenotypes across

various vertebrate species used to model binge drinking[39,99,108]. These studies show that binge drinking during pregnancy remains a formidable threat to perinatal health, and also indicate a worsening exposure trend to an exceedingly harmful drinking pattern.

For studies discussed in the subsequent chapters, a once-daily binge pattern was used in a rat model, with treatment starting after pregnancy confirmation on gestational day (GD) 5 and continuing throughout pregnancy until just prior to term (GD 21). In addition to modeling the harmful effects of binge drinking, this study design accounted for the following factors with key relevance in FASD animal model studies:

1) *Alcohol administration*. We dosed treatments via orogastric gavage to more closely mimic how humans consume alcohol. Numerous animal model FASD studies use alcohol administration methods, such as inhalation or intraperitoneal injection, that don't align with how humans orally ingest alcohol[123]. Orogastric gavage prevents irritation to mucosal surfaces (e.g., the lining of the lungs or organs in the peritoneal cavity), and it accounts for alcohol absorption and metabolism via the gastrointestinal tract (i.e., presystemic metabolism)[123–125]. A more relevant but less applied method in animal model studies is a voluntary drinking paradigm, but very few species will consume alcohol willingly. Notable exceptions are pigs and primates, but despite being analogous to humans in many ways, these models are costly and labor intensive. Selective breeding has produced mice strains to be fairly reliable drinkers, however, rarely will they drink to the point of excess[123,126]. Gavage ensures precise delivery of alcohol and target BACs are reached, and it allows for caloric intake to be more closely matched between treatment groups, thus minimizing nutritional discrepancies[123,125,126]. Prior work indicates that chronic orogastric gavage administration (SID q 28 days) in Sprague–Dawley rats does

not produce more stress than restraint handling alone and is well tolerated with no significant biological stress[127]. For our studies, flexible polyurethane tubes (Instech Laboratories, Inc., 13ga, FTPU-13-88-50) were used instead of conventional stainless-steel ball-point gavage 'needles' as an added safeguard to minimize stress.

2) *BAC*: Higher peak BACs occur in response to binge drinking compared to other drinking paradigms. The target mean BAC achieved in our studies was roughly 289 mg/dl and is comparable to BACs obtained in both clinical patients who binge drink and animal models of binge alcohol exposure[38,47,112,128].
3) *Exposure duration*: The chronic exposure duration used in our model is pertinent, as an estimated one third of women who drink during pregnancy will do so throughout their pregnancy[129,130]. Although alcohol administration occurs chronically throughout gestation, exposure in our rat model is limited to the first- and second-trimester equivalents of human brain development (GDs 1-10 and 11-21, respectively). This correlates with a pattern in nearly two thirds of pregnant drinkers who will drink alcohol until realization and/or progression of pregnancy[130]. Chronic drinking during pregnancy is also associated with FASD clinical cases and phenotypes[129,130].
4) *Placental interactions*: In the following studies, fetal alcohol exposure only occurs with a functioning placenta. A predominant exposure paradigm in FASD studies assessing the effects of PAE on brain and behavior is to administer alcohol directly to pups during postnatal days 1-10[113,131–135]. This developmental period aligns with the third-trimester equivalent of human brain development, and comprises the brain growth spurt, which in humans, occurs at parturition. A major disadvantage of this popular paradigm is it does

not account for the interactions that occur at the fetal-maternal interface, such as nutrient/oxygen transfer, waste exchange, and alcohol metabolism.

5) *Nutrient intake*: Heavy alcohol consumption has been shown to correspond with altered dietary intake, and conversely nutritional deficiencies can influence alcohol metabolism[136]. Both of these factors can have an effect on adverse fetal outcomes, such as low birth weight[136–138]. At the start of our studies, dams assigned to the alcohol group were matched with a similar-weight dam in the pair-fed control group. Chow consumed by alcohol-exposed dams was weighed daily, and this amount corresponded to the amount of chow given that day to its pair-fed counterpart. Pair-fed controls were also given daily treatment of 50% maltose dextrin, to account for the calories in alcohol and to control for the gavage procedure.

Studies described in the proceeding chapters utilize a well-characterized animal model and exposure pattern, allowing for comparison with a copious amount of FASD investigation and building on their foundational knowledge.

### 1.4. Jones and Smith, Revisited: Persisting Knowledge Gaps and Shifting Focus Beyond the Brain

To summarize up to this point, FASD is a lifelong disorder with no approved pharmaceutical therapy to mitigate symptoms, no clear-cut mechanistic perspicacity, alcohol's teratogenic limits have yet to be defined, and although its cause unambiguous, FASD has proven nearly impossible to prevent or even diminish due to the convoluted relationship humans have with alcohol. Moving forward, solving the FASD puzzle will require the following:

1) a comprehensive working knowledge of what has been ascertained with respect to alcohol's teratogenicity. The sheer volume of FASD work across various disciplines poses a fundamental challenge;
2) field advancement is contingent upon FASD researchers being willing to step outside the familiarity of their respective fields by and asking pertinent questions that use multidisciplinary approaches and collaborations;
3) whole animal model studies are critical for field advancement because human studies are limited by logistics and ethical concerns. These preclinical models remain imperative for mechanism discernment, treatment development, and for continuing to elucidate disease pathogenesis.

Unfortunately, as more is known about FASD pathogenesis, the less likely a silver bullet solution becomes. Deciphering the FASD puzzle poses a daunting challenge and is not an investigative field for the faint of heart or those who are easily deterred.

A good starting point for finding answers, or better yet asking the right questions, is the FASD initiatives outlined by the NIAAA in their strategic plan for 2017-2021, "While research on FASD has historically focused on the neurobiological deficits associated with the condition, the research community has become increasingly aware that prenatal alcohol exposure has wide-ranging effects on other organ systems…" This objective points back to findings in the foundational Jones and Smith paper noting cardiac anomalies in all patients, as well as in half of the patients in the case study by Streissguth and colleagues, which collectively suggest that the cardiovascular system is distinctly vulnerable to PAE. Surprisingly, this claim of cardiovascular system susceptibility to PAE remains insufficiently explored.

Cell populations and organ systems developing *in utero* are dependent on healthy cardiovascular function, as it maintains optimal nutrient and oxygen delivery, waste exchange, hormone and related cell signaling, all of which in turn support cell growth, differentiation, tissue development, and maintain homeostasis in a rapidly evolving environment. Based on the fundamentality of the cardiovascular system to fetal development, one could speculate that alcohol-induced cardiovascular damage is an underlying mediator for developmental deficits associated with FAS/FASD. Given that the developing brain and vascular system share deep interconnectivity, one could reason alternatively that the harmful effects alcohol has on the brain play a direct role in compromising vascular development. A chief example of this interconnectivity is that vascular smooth muscle cells (responsible for maintaining vascular tone) and pericytes (cells with roles in blood vessel formation, blood-brain barrier maintenance, and blood flow regulation) are derived from the neural crest during fetal development[139]. The neural crest has also been shown to give rise to the craniofacial bones and glia, two structures with profound susceptibility to PAE[140,141]. Yet in response to Jones and Smith 1973, the brain became a primary target of investigation, whereas alcohol-mediated vascular effects received less emphasis. Vascular abnormalities have proven to be valuable indicators of fetal health and predictors of adverse perinatal outcomes outside the context of FASD, (e.g., clinical assessment of pre-eclampsia and fetal growth restriction[142,143]), and adequate attention to this area in the FASD remains lacking.

In the first proceeding studies, we took take a step back to look at the bigger picture answer an even less explored question: how does alcohol affect the maternal reproductive vasculature? The maternal uterine artery serves as a lifeline to the fetal compartment, ensuring optimal nutrient and oxygen delivery for supporting rapid growth and development *in utero*. To

achieve this, it regulates perfusion through myogenic tone and undergoes profound remodeling to meet the ever-increasing metabolic demands of the fetus[144–146]. Previously, it has been shown that alcohol impairs maternal uterine artery endothelial adaptations *in vitro* and blood flow as well as uterine spiral artery remodeling *in vivo*[42,106,107,147–150]. The objectives of chapters 2 and 3 were to build upon these works, which collectively suggest that the maternal reproductive vasculature is vulnerable to alcohol during pregnancy. In Chapter 2, we hypothesized that alcohol impairs vascular adaptations in the uterine artery during pregnancy that are essential for maintaining healthy pregnancy. If alcohol compromises the maternal uterine artery's capacity to dynamically adapt to pregnancy, it is possible that the downstream effects could result in fetal harm observed in FASD. In Chapter 3, we hypothesized that alcohol impairs maternal uterine artery function by decreasing vascular relaxation in the maternal uterine artery, that alcohol-mediated damage occurred at the endothelial level, and that nitric oxide (NO) was the endothelial-derived vasodilator chiefly susceptible to binge alcohol exposure. The inability of the uterine artery to sufficiently relax in pregnancy may increase resistance which may in turn potentially diminish essential blood flow to the fetal compartment. Similar uterine arterial dysfunction has been observed in response to stressors known to compromise pregnancy (e.g., nicotine, undernutrition[151–154]), warranting further investigation in the context of FASD.

Lastly, we come to the most studied target of FASD: the brain. The brain remains an enduring focus despite extensive documentation of PAE-mediated brain damages because so much remains unknown. Exact mechanisms of alcohol-mediated damage are elusive, as are the definitive limits of alcohol-mediated brain damage. We built upon previous studies assessing regional vulnerability of the brain, which have shown that alcohol differentially affects structures in the brain with unique functions. The cerebellum, hippocampus, and cerebral cortex are FASD

regions of interest because their distinctive functions have shown susceptibility to alcohol at any stage of fetal development and because the outcomes of damage to these structures is well-recognized.

Our objective in Chapter 5 was to determine the effects of PAE on amino acid abundance in the aforementioned brain regions of interest. Amino acids are renowned as nutrients that serve as protein building blocks, but in the brain particularly they have critical roles in cell signaling. Amino acids are the most abundant neurotransmitters in the brain, and their availability is tightly regulated to maintain optimal neurotransmission. In the developing brain, amino acids are essential for moderating three candidate mechanisms for FASD brain deficits: synaptogenesis, neuronal proliferation, and migration[159]. To date, amino acid disruption has yet to be linked as an underlying contributor for these brain deficits in FASD. Excitatory amino acids in excess induce neurotoxicity, whereas amino acid depletion impairs brain functions such as cognition[160,161], and it can be reasoned that amino acid disruption in response to PAE could have profound effects on brain development. Although it has been shown alcohol alters amino acid levels in maternal and fetal plasma[116,162], this has not been assessed in the context of specific brain regions following a model of chronic binge PAE. We hypothesized that PAE alters brain amino acid concentrations in a region-specific manner and that amino acids critical roles in brain development may be disproportionately affected.

In Chapter 6, we converged on the fetal hippocampus and assessed the effect of PAE on its transcriptome via next-generation RNA sequencing (RNA-seq). Transcriptome sequencing is a rapidly evolving tool used to better understand healthy development and disease progression by providing a snapshot of the expression levels of functional genomic elements and allowing for comparison of this between healthy and diseased tissue. The method used in this study (high

throughput deep-sequencing) improves upon limits imposed by older technology that lack sensitivity and a more dynamic detection range. Previous work using primarily microarrays have shown alcohol alters gene expression in the developing hippocampus[163–166], and we hypothesized that chronic gestational alcohol exposure alters hippocampal transcriptome expression levels during fetal development as well as their related global canonical pathways.

The overall objective of this study compilation was to advance what is known about FASD to better understand the nature of the disease so that treatment approaches developed in the future may be targeted and more effective. To achieve this, we assessed how alcohol influences both less explored target organs such as the maternal uterine vasculature and placenta as well as shed new insights into how the brain responds to alcohol during development. Given that FASD remains a significant public health issue, a critical need for preclinical investigation such as the following studies persists until pathogenesis is fully understood.

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# 2. CHRONIC BINGE ALCOHOL CONSUMPTION DURING PREGNANCY ALTERS RAT MATERNAL UTERINE ARTERY PRESSURE RESPONSE*

## 2.1. Introduction

Fetal Alcohol Spectrum Disorders (FASD) refers to the range of physical, mental, functional, and/or behavioral abnormalities following developmental alcohol exposure[1–4]. The United States Surgeon General and the American Academy of Pediatrics have issued recommendations to abstain from any alcohol use during pregnancy[5–7]. Despite these repeated advisories, the CDC recently estimated alcohol use in 10.1% of pregnant women aged 18–44 and binge drinking in 3.1% of pregnant women within the years 2011–2013[8]. Among school-age children in the United States and some Western European countries the prevalence of FASD is estimated to be 2–5%[9].

In the past four decades, marked progress has been made to understand the effects of maternal alcohol consumption during pregnancy. Thus far, an extensive number of studies have focused on understanding alcohol's teratogenic role in brain & neurodevelopment[1,3,10], while little is known about its effects on the mother, specifically the maternal uterine artery which delivers all the nutrients and oxygen to the developing fetus and its supporting tissues. We herein focus on the effects of binge alcohol exposure on remodeling of the maternal uterine artery. During normal pregnancy, the maternal uterine artery undergoes outward hypertrophic

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remodeling, characterized by an increased vessel cross sectional area and a decreased ratio of medial thickness to lumen diameter or media:lumen ratio. Systemic vascular adaptations to pregnancy include increases in vascular compliance and a decrease in mean arterial blood pressure[11–13]. These changes to the maternal vasculature are essential for a healthy pregnancy outcome.

We and others have reported that alcohol consumption during pregnancy results in altered uterine endothelial adaptations[14–17], agonist-dependent vessel relaxation[18], spiral artery remodeling[19], and blood flow[20]. However, there exists no study on alcohol-related myogenic response, a critical gestational uterine vascular adaptation. In response to changes in the transmural pressure, the uterine artery will react by dilating or constricting to alter the resistance in order to maintain the perfusion rate, an intrinsic adaptation called myogenic reactivity[11,21–23]. The aim of this study was to investigate pressure-dependent maternal uterine artery responses and vessel remodeling parameters following chronic binge alcohol exposure during pregnancy.

## 2.2. Materials and Methods

### 2.2.1. Treatment Groups and Dosing Paradigm

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at the Texas A&M University. Rats were housed in a temperature-controlled room (23 °C) with a 12:12-h light–dark cycle. Timed pregnant Sprague–Dawley rats were purchased from Charles River (Wilmington, MA). Two groups were utilized including a nutritional pair-fed control and a binge alcohol group. The number of animals in the pair-fed control and alcohol groups were n = 11 and n = 12 respectively. Uterine arteries that were either

mechanically damaged during tissue preparation for pressure myography or did not show any functional response by constricting at 60 mm Hg were not studied, and the number of these vessels was not different between treatment groups[24,25]. Maternal uterine arteries from a total of n = 8 rats in each group exhibited pressure-dependent vascular response by constricting at 60 mm Hg and were all analyzed. Dams in the alcohol group received a binge-like-dose of 6 g/kg body-weight/day of 28.5% w/v, ethanol via oral gavage from GD 5-19[26,27]. The regimen of exposure utilized in this study is based on reported alcohol consumption patterns in pregnant women and FASD animal models[26–31]. Daily feed intake of the alcohol rat was measured. The pair-fed control dams were matched with alcohol-fed dams of similar weight, and food intake was measured daily and yoked[32]. Pair-fed control animals were also gavaged daily with isocaloric maltose-dextrin to control for the calories derived from alcohol. There was no significant maternal weight difference between the groups on GD 20 (Pair-fed controls, 310 ± 8 g; Alcohol, 305 ± 7 g). Animals were sacrificed on GD 20, one day after the last alcohol exposure.

### 2.2.2. Tissue Preparation

Following sacrifice, the whole uterus was transferred to a large 200 mm petri dish filled with solidified Sylgard™ containing cold HEPES – Bicarbonate Solution (pH 7.4; NaCl 130 mM; KCl 4 mM; $MgSO_4 \cdot 7H_2O$ 2.5 mM; $NaHCO_3$ 4.05 mM; $CaCl_2$ 2.4 mM; HEPES 10 mM; $KH_2PO_4$ 1.18 mM; Glucose 6 mM; EDTA 0.024 mM), where it was pinned to facilitate the cutting of the uterine artery. Primary uterine artery of approximately 3–5 mm was cut between bifurcations and washed in HEPES-Bicarbonate Solution, to remove excess fat and connective tissues. A dual-chamber arteriograph was used for our experiments, which permits vessels from a

control and an alcohol-fed dam to be studied consecutively under identical experimental conditions.

### 2.2.3. Pressure Myography

A dual-chamber pressure myograph system (Living Systems Instruments, VT) was used for the experiments. The chamber consisted of an inflow and an outflow port to maintain the bath level such that the vessel remained submerged under the superfusate of warmed HEPES buffer. There were two glass cannulas on opposite sides of the chamber. The vessel was mounted on these cannulas positioned above a cover glass from where it was visualized utilizing an inverted microscope (Accu-Scope) mounted with a CCIR camera (IonOptix Corporations, MA). One of the cannulas was connected to a pressure transducer, which converted fluid pressure into an electrical signal and sent this data to the pressure servo controller. Based on the output value, the pressure servo controller in-turn sent signal to the pressure pump to maintain the set pressure. The pressure was observed with the pressure monitor and was recorded with IonWizard software (IonOptix Corporations, MA). Simultaneously, the camera sent the image to the video dimension analyzer software (IonWizard, IonOptix Corporation, MA) which calculated various parameters such as vessel diameter, lumen diameter, media:lumen ratio, etc.

The chambers, cannulas, tubing, and stopcocks were primed with HEPES buffer prior to mounting the vessel, to ensure no air passed into the vessel. Each vessel was transferred to one of the two chambers of the dual-chamber pressure myograph system. Using a dissection microscope, the endothelium-intact vessel was then mounted on the glass cannulas that were connected to the pressure transducer and tied using two nylon ligatures. Excess blood was removed from inside the vessel by slowly injecting buffer through the stop valve using a syringe

attached to a 45 μm syringe filter. The opposite end was tied off using a nylon ligature, which was in-turn ligated to the opposite glass cannula. The chamber was placed on the inverted microscope to complete the setup described above (Fig. 1). The pressure transducer tubing was filled with warm 37°C HEPES buffer, and were connected via stopcocks to the cannulas, creating a closed-pressure system.

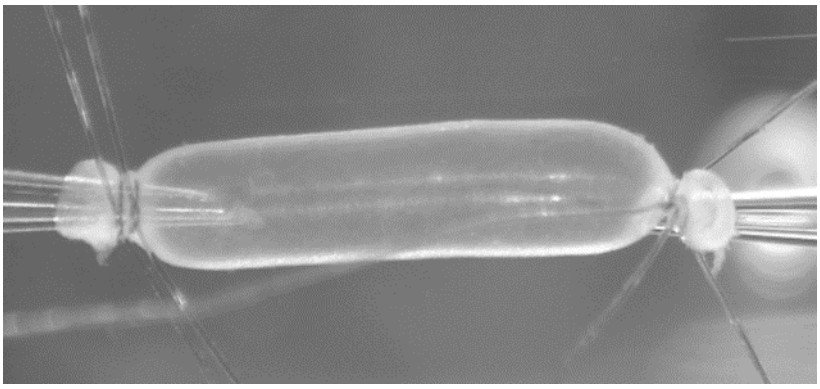

**Figure 1:** *Cannulated and pressurized uterine artery*. Representative picture of the maternal uterine artery (gestational day 20) mounted in a pressure arteriograph.

### 2.2.4. Experimental Protocol and Data Analysis

Transmural vessel pressure was gradually increased and set to 60 mm Hg using the pressure servo controller. The vessels were allowed to equilibrate in the 37°C superfusate buffer for 60 min, or until the vessels started showing myogenic tone[33]. The pressure was then lowered to 20 mm Hg, where constriction was inhibited[11], and baseline measurements of vessel diameter,

lumen diameter, and media:lumen ratio were recorded. The diameter was considered stable when the constrictions stabilized or there were no detectable changes in measured variables (3 min–5 min). Subsequently, the pressure was increased by 20 mm Hg, starting 40 mm Hg until the pressure reached 120 mm Hg. After the measurements were complete, the data were transferred to a computer for analysis. The maximal contracted diameters at the stabilized state were determined for each pressure. The percent change from the baseline was analyzed by a two-way ANOVA with the treatment group as the between factor and the pressure as the within factor. Further pairwise comparisons were performed when appropriate using Fisher's protected LSD. Level of significance was established a priori at $P < 0.05$.

**2.3. Results**

The dual-chamber arteriograph system described above is graphically represented (Fig. 1). To illustrate the pressure-dependent response of a uterine artery, an experimental tracing from a control and an alcohol-fed pregnant dam is depicted in Fig. 2. The maternal uterine artery diameter stabilized at 20 mm Hg, showed passive distension with increase in pressure to 40 mm Hg, and redeveloped tone at 60 mm Hg and was maintained until 120 mm Hg.

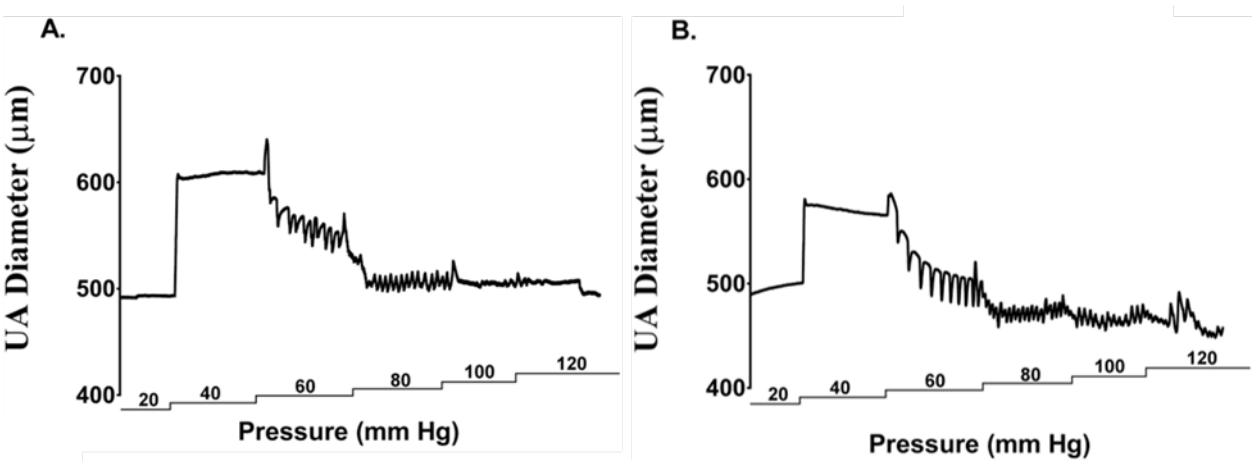


**Figure 2:** *Uterine artery (UA) pressure arteriograph traces from (a) pair-fed control and (b) alcohol dams*. The maternal uterine artery diameter stabilized at 20 mm Hg, showed passive distension with increase in pressure to 40 mm Hg, and maintained tone between 60 mm Hg and 120 mm Hg.

In both control and alcohol treatment groups, maternal uterine artery exhibited pressure-dependent myogenic constriction as pressure was increased from 40 mm Hg (Fig. 3). The percent change in the uterine artery vessel diameter from baseline showed a significant main effect of pressure in both the control and alcohol groups (pressure effect, $P < 0.0001$). There was also a main effect of alcohol on the percent constriction of the uterine artery vessel diameter (alcohol effect, $P = 0.0025$); the alcohol group exhibited significantly greater pressure-dependent constriction compared to the controls.

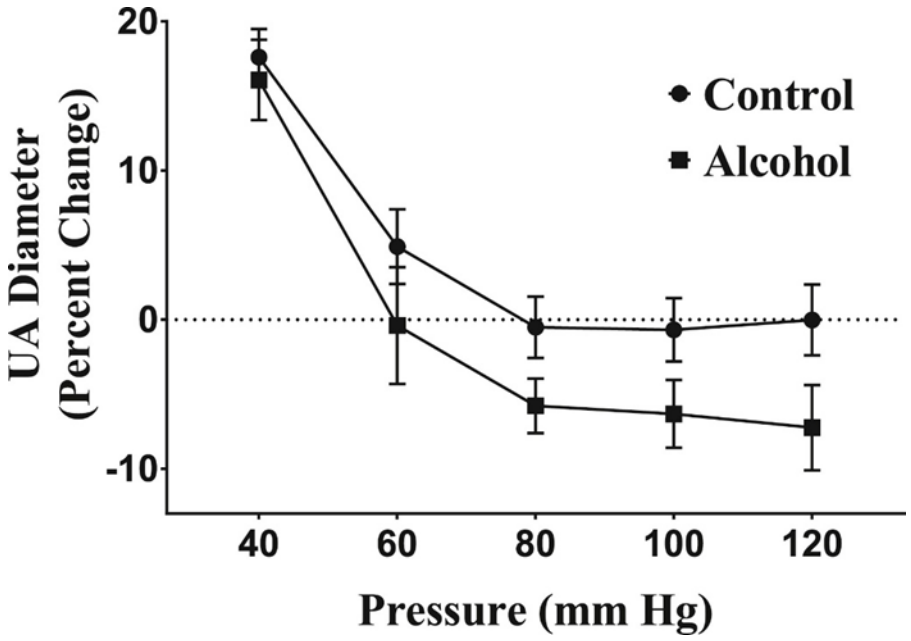


**Figure 3:** *Effect of alcohol on percent change of uterine artery (UA) diameter with pressure*. Percent change (from baseline) in uterine artery vessel diameter was significantly greater in the alcohol group compared to the control group and the alcohol group exhibited greater pressure-dependent constriction (pressure effect, $P < 0.0001$; alcohol effect, $P = 0.0025$).

The uterine artery lumen diameter displayed a significant main effect of pressure in both control and alcohol groups (pressure effect, $P < 0.0001$; Fig. 4). Similar to the uterine artery vessel diameter, percent change in uterine artery lumen diameter was significantly greater in the alcohol group; the alcohol group exhibited greater pressure-dependent constriction of lumen diameter compared to the control group (alcohol effect, $P = 0.0020$).

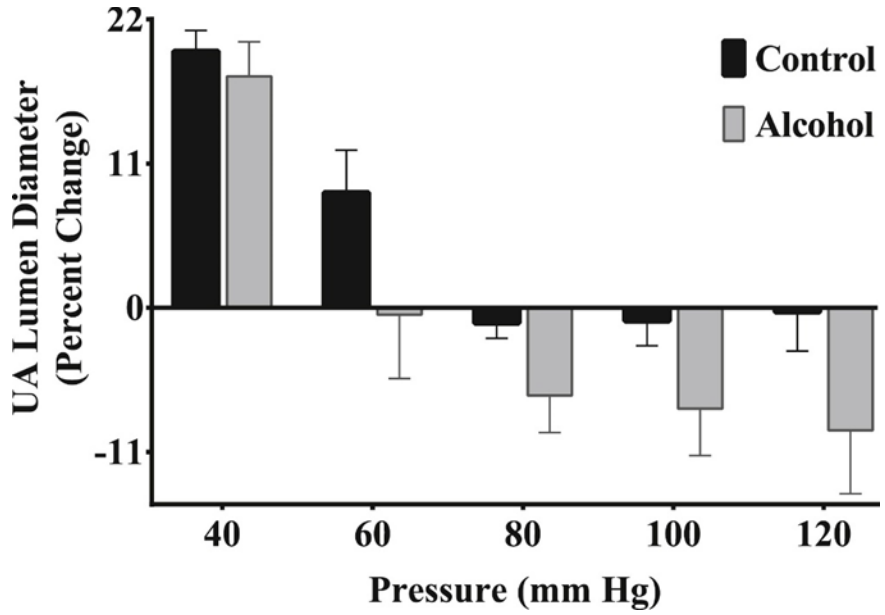


**Figure 4:** *Effect of alcohol on percent change of uterine artery (UA) lumen diameter with pressure*. Percent change (from baseline) in uterine artery lumen diameter was significantly greater in the alcohol group; alcohol group exhibited greater pressure-dependent constriction of lumen diameter compared to the control group (pressure effect, $P < 0.0001$; alcohol effect, $P = 0.0020$).

The percent change in the media:lumen ratio, an indicator of vessel remodeling, was significantly higher in the alcohol group compared to the controls (alcohol effect, $P < 0.0001$; Fig. 5). Both the control and the alcohol groups displayed a significant main effect of pressure (pressure effect, $P < 0.0001$).

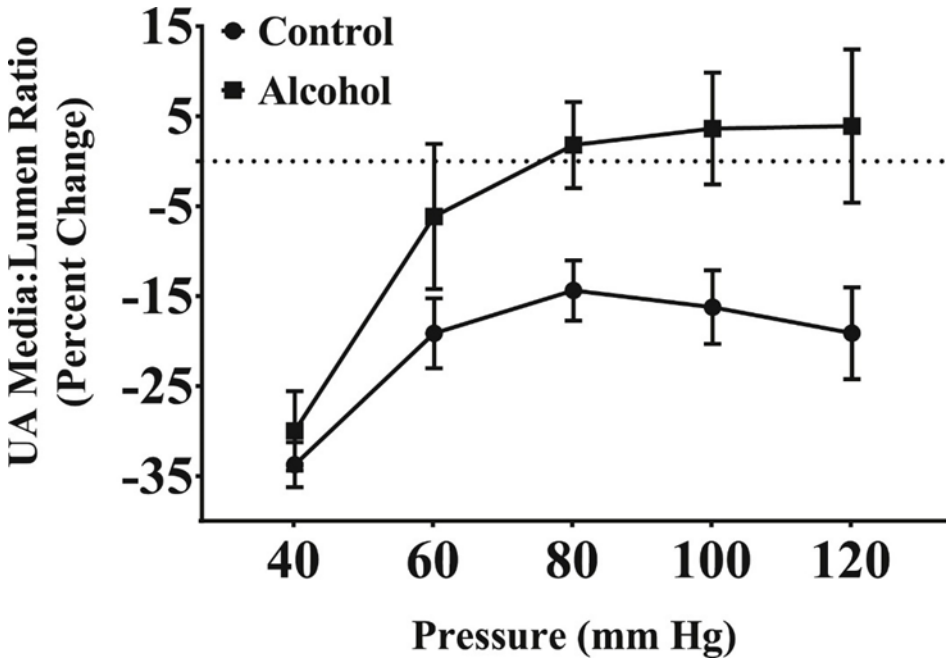


**Figure 5:** *Effect of alcohol on percent change of uterine artery (UA) media:lumen ratio*. Percent change in uterine artery media:lumen ratio was significantly higher in the alcohol group compared to the control group (pressure effect, $P < 0.0001$; alcohol effect, $P < 0.0001$).

## 2.4. Discussion

The purpose of this study was to investigate the functional response of the maternal uterine artery to changes in intra-luminal pressure following chronic binge alcohol exposure during pregnancy. Three salient findings can be gleaned from the current study: First, gestational alcohol exposure results in greater pressure-dependent uterine artery constriction. Second, uterine artery vessel/lumen diameters and the media:lumen ratio together indicate gestational alcohol-induced inward hypotrophic remodeling. Third, our overall findings suggest that *in vivo* binge-alcohol exposure leads to altered gestational uterine arterial adaptations.

In this study, both the excised uterine arteries of control and alcohol-fed rats exhibited decreases in the uterine artery diameter as the intraluminal pressure was increased from 40 mm Hg. However, compared to the controls, gestational alcohol exposure resulted in greater pressure-dependent uterine artery constriction, and the effect was most pronounced at physiologic pressures of 80 mm Hg–120 mm Hg. Interestingly, we observed that the uterine arteries of the control group maintained their diameter throughout this range of pressures, whereas the alcohol group exhibited lower vessel diameter at the same pressures. We can deduce using Poiseuille's equation that resistance is inversely proportional to the radius to the fourth power, and thus changes in diameter observed in our study can lead to large significant decreases in uterine blood flow. These data are consistent with previous reports demonstrating ~40% decrease in uterine blood flow (UBF) following chronic binge alcohol exposure in sheep[34]. Chronic alcohol-induced UBF decrease of about ~40% would lead to a reduction in the uterine artery radius to normalize the shear, as noted in our study. Previous studies demonstrate that uterine artery myogenic constriction is decreased in pregnancy compared to non-pregnant mice and sheep[35,36], whereas others have shown increased myogenic reactivity in myoendometrial arteries of rabbits and radial uterine arteries of rats[37,38]. Although the current manuscript is the first to report alcohol-induced myogenic constriction, other rat model studies investigating developmental insults have demonstrated that maternal undernutrition during pregnancy resulted in significantly increased myogenic tone in radial uterine arteries on GD 20 when compared with control[25]. It is well established that myogenic adaptations are important during pregnancy to regulate blood flow to the feto-placental compartment. Collectively, our data suggest that alcohol-induced uterine artery adaptations may contribute to altered hemodynamics in the utero-placental circulation which are critical for delivery of gas and nutrients[35,39].

We herein demonstrate that the uterine arteries of the alcohol-administered rats undergo inward hypotrophic remodeling, as measured by a decrease in lumen diameter and an increased media:lumen ratio[40]. This is in contrast to normal pregnancy uteroplacental vascular adaptations, which is associated with outward-hypertrophic remodeling, i.e., an increased lumen diameter and a decreased media:lumen ratio, resulting in decreased resistance and increased blood flow[13,41,42]. An increase in media:lumen ratio corresponds to increased resistance to blood flow[43,44]. Future studies on elastin, collagen, and smooth muscle remodeling are warranted to further characterize alcohol-induced uterine artery programing.

Our overall findings suggest that in vivo binge-alcohol exposure leads to altered gestational uterine arterial adaptations. During a healthy pregnancy, gestation-induced 30–50-fold increase in uterine blood flow is critical to meet the developmental requirements of the growing fetus[42]. In women, uterine vascular resistance and resistance index decrease from 1.93 ± 0.22 mm Hg $ml^{-1}$ min and 0.89 in the non-pregnant state to 0.14 ± 0.01 mm Hg $ml^{-1}$ min and 0.52 by 34 weeks of gestation, respectively[45,46]. Furthermore, animal models employing microspheres have validated that cardiac output to the uterus increases from 0.5% in non-pregnant state to 7.7% and 15.7% by the $2^{nd}$ and $3^{rd}$ trimester-equivalents of pregnancy, respectively[41,42]. The aforementioned uterine vascular adaptations are essential for guaranteeing the nutrient requirements of the fetus be met throughout pregnancy and thus for normal fetal growth and development[41,47,48]. Our current findings of alcohol-induced altered uterine artery adaptations are supported by earlier work on the impact of alcohol on uterine vasculature, specifically uterine artery endothelial mRNA[49] and protein profile[15], endothelial derived vasodilatory pathways[50], endothelial cell proliferation[51], and spiral artery remodeling[19].

From the current study and others, it is evident that chronic binge alcohol exposure disrupts maternal uterine artery adaptations during pregnancy. The underlying mechanisms of alcohol-mediated pressure-dependent vascular response of the maternal uterine artery remain to be determined. Alcohol consumption during pregnancy is linked with intrauterine growth restriction and low birthweight[52], hallmark features of Fetal Alcohol Syndrome, and we hypothesize that maladaptive changes to uterine circulation may play a causal role in these abnormalities. Thus, we aim to continue to explore the consequences of alcohol exposure on the maternal vascular compartment so that we may better understand how these changes impact the fetus.

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# 3. MECHANISMS UNDERLYING CHRONIC BINGE ALCOHOL EXPOSURE-INDUCED UTERINE ARTERY DYSFUNCTION IN PREGNANT RAT*

## 3.1. Introduction

Maternal alcohol exposure is strongly associated with a wide array of irreversible damage to the developing fetus, which can manifest as a range of physical and functional abnormalities, termed fetal alcohol spectrum disorder[1]. Recent reports estimate the prevalence of FASD to be between 2 and 5% in young school-aged children in both the United States and Western European countries[2]. The most severe form of FASD, fetal alcohol syndrome (FAS), was first defined as a pattern of altered growth, morphogenesis, and behavior in offspring of chronic alcoholic mothers[3]. The cost burden of FAS in the United States was estimated to be $6.5 billion in 2010[4]. Much of the emphasis within the FASD field remains directed toward understanding alcohol-mediated brain and behavioral deficits[5]. Despite significant advances in discernment of neuropathogenesis, the mechanism(s) underlying gestational alcohol-mediated growth deficits remain essentially unknown. We reason that developmental deficits associated with gestational alcohol exposure may emanate from alcohol-induced alteration(s) to a mother's natural physiological adaptations associated with normal healthy pregnancy. Specifically, we focused this study on alcohol-induced alteration(s) to maternal uterine vascular adaptations that occur during pregnancy.

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During a normal pregnancy, dramatic adaptive changes to the maternal primary uterine artery ensure optimal nutrient and oxygen delivery for meeting the demands of fetal growth, development, and metabolism. In women, the primary uterine artery accomplishes this by markedly increasing its diameter compared to the nonpregnant state (1.4 mm to 3.4 mm by third trimester of pregnancy), which in turn corresponds to a striking decrease in uterine vascular resistance[6,7]. Alcohol has been reported to disrupt uterine arterial adaptations [8], including blood flow in sheep[9] and spiral artery remodeling in rats[10]. In this study, we sought to examine the mechanism by which alcohol affects pregnancy-induced uterine vascular adaptation, specifically agonist-induced uterine vascular reactivity. Uterine artery vasodilation is mediated by 3 key endothelial-derived vasodilators: nitric oxide (NO), prostacyclin (PGI2), and endothelial-derived hyperpolarizing factor (EDHF)[8]. NO has been pivotally implicated in pregnancy, as prior works report both systemic and uterine artery-specific increases in NO production during pregnancy in humans[11,12], and in various animal models such as rats[13] and sheep[14]. Interestingly, although a substantial body of work investigating *in vitro*, human, and animal models indicates that alcohol alters vascular NO production[15,16], knowledge of this remains limited in the context of gestational alcohol exposure. One study has shown that alcohol decreased pregnancy-specific, NO-mediated vasodilation of the mesenteric artery in pregnant mice[15]. Although some investigation has explored the effect of gestational alcohol exposure on NO-mediated vasodilation in the systemic vasculature, alcohol's impact on the reproductive vasculature, specifically the uterine artery, which primarily delivers oxygen and nutrients to the fetus, has yet to be established.

Endothelial nitric oxide synthase (eNOS) is enzymatically responsible for constitutive vascular NO production, and its activity is regulated by multiple posttranslational

modifications[17,18]. One of the most essential and widely studied eNOS posttranslational modifications is the phosphorylation of serine at eNOS position 1177 (P-Ser$^{1177}$eNOS), which directly leads to increased eNOS enzymatic activity[19]. Previous *in vitro* investigations of uterine artery endothelial cells derived from pregnant sheep and human umbilical endothelial cells[20,21] have determined alcohol alters phosphorylation of Ser$^{1177}$eNOS. Following the previous study by our group which determined alcohol-induced vascular dysfunction of the uterine artery using wire myography[21,22], the current study assesses the mechanisms underlying *in vivo* alcohol exposure-induced uterine artery dysfunction in a pressurized vessel. We hypothesize that chronic binge alcohol exposure during pregnancy will decrease endothelial-dependent vascular relaxation in the maternal uterine artery by impairing NO-mediated vasodilation via alteration of eNOS posttranslational modification.

## 3.2. Materials and Methods

### 3.2.1. Treatment Groups and Alcohol-Dosing Paradigm

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at the Texas A&M University and University of Texas Medical Branch. Timed-pregnant Sprague Dawley rats purchased from Charles River (Wilmington, MA) were housed in a temperature-controlled room (23°C) with a 12-hour:12-hour light–dark cycle. For dual-chamber arteriograph experiments, 2 treatment groups were utilized, comprised of a nutritional pair-fed control group and a binge alcohol group. An additional untreated normal control group was included in immunoblotting studies (detailed in immunohistochemistry section). Dams in the alcohol treatment group acclimatized via a once-daily, binge-like, orogastric gavage dose of 4.5

g/kg ethanol (EtOH) (22.5% wt/v; peak blood alcohol concentration [BAC], 216 mg/dl) from gestational day (GD) 5 to 10 and progressed to a 6 g/kg alcohol dose from GD 11 to 20 (28.5% wt/v; peak BAC, 289 mg/dl)[23,24]. To control for nutrition, daily feed intake of the alcohol rat was measured, and the pair-fed dams received an equal amount of feed as its weight-paired alcohol animal. Isocaloric maltose–dextrin was given once-daily to the pair-fed control rats to equate the calories derived from administered alcohol. The exposure regimen utilized in this study was modeled after reported alcohol consumption patterns in pregnant women and FASD animal models[23,25–27].

### 3.2.2. Maternal, Fetal, and Placental Weight Measurements

Maternal weights of pregnant pair-fed control (n = 8) and alcohol (n = 8) rats were measured on GD 20, 1 day after the last EtOH administration on GD 19. Following euthanasia of the dam, the concepti were carefully removed and each fetus and placenta were separated and weighed individually.

### 3.2.3. Endothelial-Mediated Vasodilation of the Uterine Artery

Functional uterine artery studies were conducted in a separate set of rats. Pregnant pair-fed control (n = 8) and alcohol-administered (n = 8) rats were utilized to study the functional response of the primary uterine artery. As previously described[28], following euthanasia, the whole uterus was transferred to a large 200-mm petri dish containing solidified Sylgard and filled with ice-cold HEPES–bicarbonate solution (pH 7.4; NaCl 130 mM; KCl 4 mM; $MgSO_4 \cdot 7H_2O$ 2.5 mM; $NaHCO_3$ 4.05 mM; $CaCl_2$, 2.4 mM; HEPES 10 mM; $KH_2PO_4$ 1.18 mM; glucose 6 mM; EDTA 0.024 mM), where it was pinned to facilitate uterine artery dissection. A segment

of primary uterine artery (approximately 3 to 5 mm) was excised between bifurcations, trimmed of surrounding fat and connective tissue, and washed in HEPES–bicarbonate solution. Standard procedures for a dual-chamber arteriograph setup were utilized as described previously[29]. In brief, cleaned arterial segments from each group were mounted and secured via 2 nylon ligatures onto a glass cannula in a dual-vessel arteriograph chamber, which then was connected to a pressure transducer. Residual blood was removed from the vessel lumen, and the free vessel end was ligated and securely fastened to the opposing cannula with nylon filament. Both chambers of the arteriograph were continuously circulated with HEPES buffer fixed at 37°C, and intraluminal pressure was maintained and monitored using a pressure servo controller and a pressure monitor, respectively. This setup advantageously permitted vessels from pair-fed control and alcohol-exposed dams to be studied consecutively, under uniform experimental conditions. Following cannulation and ligation, intraluminal pressure was increased to 60 mm Hg, at which pressure the vessel exhibited myogenic tone and then equilibrated for 1 hour. After equilibration, intraluminal pressure was increased to 90 mm Hg to mimic in vivo-like conditions, at which pressure all data were recorded using Ion Wizard 6.6 (IonOptix, Westwood, MA) software. Vessels were precontracted with $10^{-7}$ M thromboxane (Tbx) for 20 minutes. Tbx concentration for this study was determined from the concentration–response curve previously generated in our laboratory[22]. Tbx treatment was followed by administration of 3-fold increasing doses of acetylcholine (Ach) from $10^{-10}$ M to $10^{-5}$ M, and the corresponding dose–response was obtained. At each dose increment, data were recorded for 3 to 5 minutes or until variable measurement remained constant.

### 3.2.4. NO-Mediated Vasodilation of Uterine Artery

Following measurement of endothelium-mediated vasodilation, we examined the NO-mediated vascular relaxation. Endothelial cells produce NO by catalyzing l-arginine to l-citrulline. $PGI_2$ is produced by sequential oxidation of arachidonic acid by enzyme cyclooxygenases (COX-1 and COX-2) and elicits smooth muscle relaxation via cyclic AMP activation, whereas EDHF relaxes smooth muscle through hyperpolarization resulting from opening of $Ca^{2+}$-activated $K^+$ channels[30]. We selectively blocked the $PGI_2$- and EDHF-mediated vascular relaxation pathways in the primary uterine artery while preserving the intact NO pathway. Uterine arteries were washed in circulating fresh HEPES buffer for 15 minutes followed by a 20-minute incubation in an inhibitor cocktail containing indomethacin ($10^{-5}$ M), a nonselective COX-1 and COX-2 inhibitor enzyme, and apamin ($10^{-7}$ M) and TRAM-34 ($10^{-7}$ M), which cooperatively inhibit $Ca^{2+}$-activated $K^+$ channels. The vessel was again constricted by circulating $10^{-7}$ M Tbx, and vascular reactivity was recorded for increasing cumulative doses of Ach from $10^{-10}$ M to $10^{-5}$ M.

### 3.2.5. Immunoblotting

As we were not limited to 2 treatment groups for this protocol as in dual-chamber arteriography, an additional untreated normal control group was also utilized with the pair-fed control and alcohol treatment groups. The untreated normal control group received ad libitum food, the alcohol group received 6 g/kg EtOH as described in the alcohol-dosing paradigm, and the pair-fed groups received isocaloric maltose–dextrin, from GD 5 to 19, until the day of sacrifice. Following sacrifice on GD 20, uterine arteries were cleaned, dissected, flash frozen in liquid nitrogen, and stored at −80°C until the day of use. The arteries were homogenized and

sonicated in 80 to 100 µl of ice-cold RIPA buffer, pH 7.5 (220 mM Tris-HCl, 150 mM NaCl, 1 mM Na2, EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin; Cell Signaling Technologies, Danvers, MA, #9806), and immediately placed on ice. Following centrifugation, the supernatant was carefully removed and stored in fresh 1.5-ml tubes. Protein concentration measurement was performed using Pierce™ BCA protein assay kit instructions (Thermo Scientific, Waltham, MA). Twenty micrograms of protein from each sample were loaded onto a 4 to 20% Tris–HEPES polyacrylamide gel (150V, 40 minutes; Mini protein II; Bio-Rad, Hercules, CA) alongside a full-range rainbow marker (GE Healthcare Bio-Sciences, Pittsburgh, PA). The proteins were then transferred onto a PVDF membrane, which was used for probing P-Ser$^{1177}$eNOS (1:1,000; #9571), β-actin (1: 5,000; #4967; Cell Signaling Technologies) as loading control, and total eNOS (1:3,000; #610297; BD Biosciences, San Diego, CA). Densitometry analysis was performed with AzureSpot software (Azure Biosystems, Dublin, CA).

### 3.2.6. Immunofluorescence

Maternal uterine arteries from random pregnant pair-fed control and alcohol rats were isolated on GD 21, flash frozen in Tissue-Tek optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA), and stored at −80°C until sectioning. Eight micrometer sections were cut using a Leica CM1860 cryostat (Leica Biosystems, Inc., Buffalo Grove, IL), transferred onto positively charged microscope slides, and stored in a −80°C freezer until undergoing immunostaining. For staining, frozen sections were fixed consecutively with ice-cold methanol (30 minutes, −20°C) and 4% paraformaldehyde (30 minutes, 4°C), rinsed in phosphate-buffered saline (PBS), incubated in 10% normal serum (1 hour), and incubated overnight (4°C)

with either a primary antibody (1:200; P-Ser1177eNOS; Cell Signaling Technology; #9571 and 1:200; total eNOS, BD Biosciences; #610297) or PBS (negative control) in a humidified chamber. The next day, the tissues were incubated in goat anti-rabbit or goat anti-mouse secondary antibody (1:250; IgG Alexa 488; Invitrogen, Carlsbad, CA; #A11008 or #A11001, respectively) for 1 hour at room temperature in the dark. After washing with PBS, 1 drop of antifade mounting media with DAPI (Invitrogen; #P36931) was applied to each slide, which were then coverslipped. Slides were again incubated overnight and were imaged the next day with a Zeiss Axioplan 4 fluorescent microscopy system using AxioVision 4.8 software (Carl Zeiss Microscopy LLC, Thornwood, NY).

### 3.2.7. Statistics

Maternal, fetal, and placental weights and placental efficiency were analyzed using Student's t-test. Vascular functional response data were analyzed using a 2-way repeated-measures analysis of variance (ANOVA), with treatment group as the between factor and dose as the within factor, followed by analysis with Sidak's multiple comparison test. Immunoblotting data were analyzed utilizing 1-way ANOVA, followed by post hoc analysis with Fisher's LSD. All data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and are presented as mean ± SEM, with significance established a priori at $p < 0.05$.

## 3.3. Results

Maternal body weight was not different ($p = 0.1955$) between the pair-fed control and alcohol-fed rats (Fig. 6). However, average fetal weight in the alcohol-administered rats (2.12 ± 0.07 g) was significantly lower ($p = 0.0015$) compared to that in the pair-fed control group (2.49

± 0.06 g), an approximately 16% weight reduction. The observed fetal growth restriction depicts one of the hallmarks of FAS commonly used in diagnosis[3].

Placental weight was not significantly different in alcohol-administered rats (p = 0.2386). Placental efficiency, calculated as fetal weight in grams divided by its respective placental weight in grams, which is an indirect measurement to calculate the efficiency by which placenta transfers nutrients to the fetus[31], was also not different between the 2 groups (p = 0.1141).

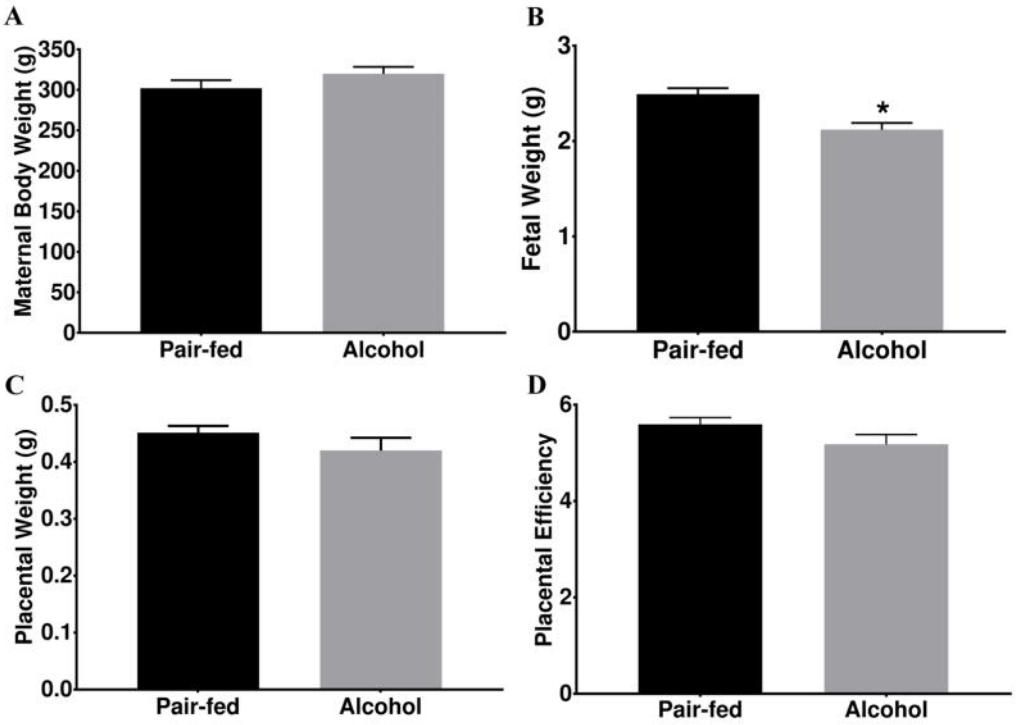


**Figure 6:** *Effect of chronic binge alcohol exposure on fetal development.* Maternal weight, fetal weight, and placental weight were measured on GD 20, one day after last alcohol exposure. Prenatal alcohol exposure **A)** did not affect mean maternal weight (P = 0.1955), however it significantly decreased **B)** mean fetal body weight (P = 0.0015). **C)** Mean placenta weight and D) placental efficiency (the ratio of fetal to placental weight) was not different between groups (P = 0.2386 and P = 0.1141, respectively).

### 3.3.1. Alcohol Significantly Decreased Uterine Artery Functional Response in a Pressurized Vessel

Uterine arteries from the pair-fed control and alcohol-administered rats were mounted on the pressure arteriograph system, and an Ach dose–response was noted under identical conditions. A comparative illustration of dose-dependent vasodilatory response is depicted in Fig. 7A. Maximal percent relaxation of the maternal uterine artery with increasing Ach dose administration was significantly lower in the alcohol group compared with that in the pair-fed control group (Fig. 7B). At the highest Ach dose ($10^{-5}$ M), uterine arteries of the alcohol-exposed rats exhibited ~30% decrease in endothelium-dependent vasodilation. This indicates a significant impairment of the uterine artery endothelial function following gestational alcohol exposure. In a separate set of pregnant pair-fed control (n = 6) and alcohol-administered (n = 6) rats, we observed no alcohol effect on endothelium-independent, sodium nitroprusside (SNP)-induced smooth muscle relaxation (Fig. 7E).

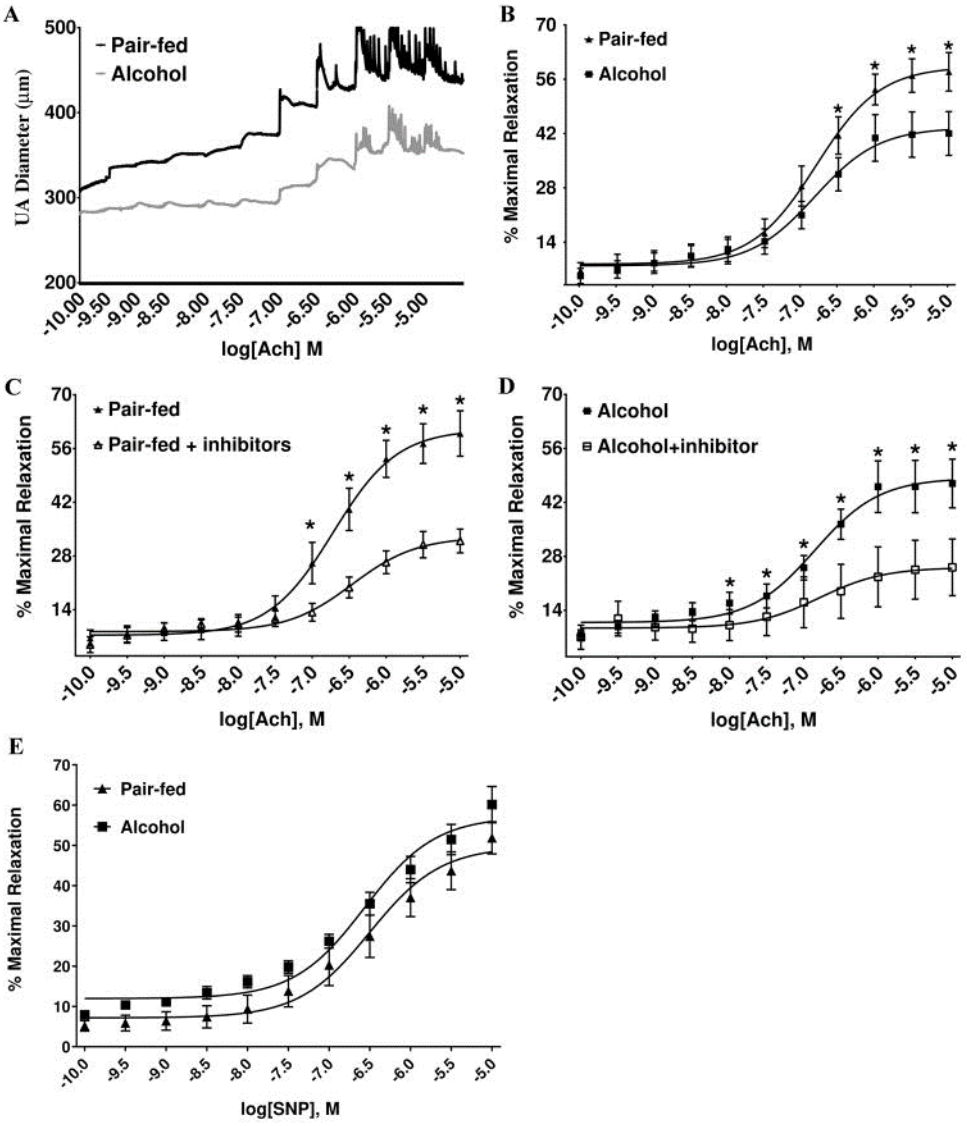


**Figure 7:** *Binge alcohol exposure during pregnancy attenuates NO-mediated vasodilation of the uterine artery in pregnant rats.* **A)** Representative pressure arteriograph tracing showing a dose-dependent vasodilatory response (uterine artery diameter, Y-axis) of the pregnant rat uterine artery with cumulative increasing doses of Ach (X-axis). **B)** Endothelial-dependent vasodilation to Ach in uterine artery from pair-fed control (▲), and alcohol-administered rats (■). To exclusively study the NO-mediated vasodilation of the rat uterine artery, EDHF and PGI2 pathways were blocked while the NO pathway was kept intact. **C)** Relaxation of the uterine artery isolated from pair-fed rats with (△) and without (▲) inhibitors, and **D)** relaxation of the uterine artery isolated from the alcohol-administered rats with (□) and without (■) inhibitors. **E)** Endothelium-independent vasodilation to SNP in uterine artery from pair-fed control (▲), and alcohol-administered rats (■). Data are expressed as mean ±SEM, of percent relaxation from maximal constricted state at 90 mm Hg.

### 3.3.2. Alcohol Specifically Altered NO-Mediated Uterine Artery Functional Response

To distinguish the pathway(s) by which alcohol alters uterine artery vasodilation, vessels were incubated with EDHF and PGI2 pathway inhibitors simultaneously. This treatment selectively preserved the NO pathway to remain intact. Following the incubation period, we assessed the vasodilatory response of these vessels to increasing cumulative Ach doses. As expected, uterine arteries from both the pair-fed control and alcohol treatment groups exhibited a significantly reduced vasodilatory response after inhibition (Fig. 7C, D). Mean vasodilation in the pair-fed control group decreased by ~46%. Interestingly, vasodilation in the alcohol-treated group decreased similarly by ~48%, which was in addition to the initial ~30% decrease prior to EDHF/$PGI^2$ inhibition. Together, these results suggest that the impaired vascular relaxation observed with gestational alcohol treatment was due to NO disruption.

### 3.3.3. Alcohol Significantly Altered Ser1177eNOS Phosphorylation Levels

#### 3.3.3.1. Immunoblotting Analysis

The phosphorylation level of excitatory $Ser^{1177}$eNOS significantly decreased in uterine arteries of alcohol-administered rats compared to those of untreated normal as well as pair-fed controls (Fig. 8). There was also a significant decrease in uterine artery total eNOS expression in the alcohol-administered rats compared with those in untreated normal and pair-fed control rats. Protein expression of β-actin, used as a loading control, was not different among treatment groups.

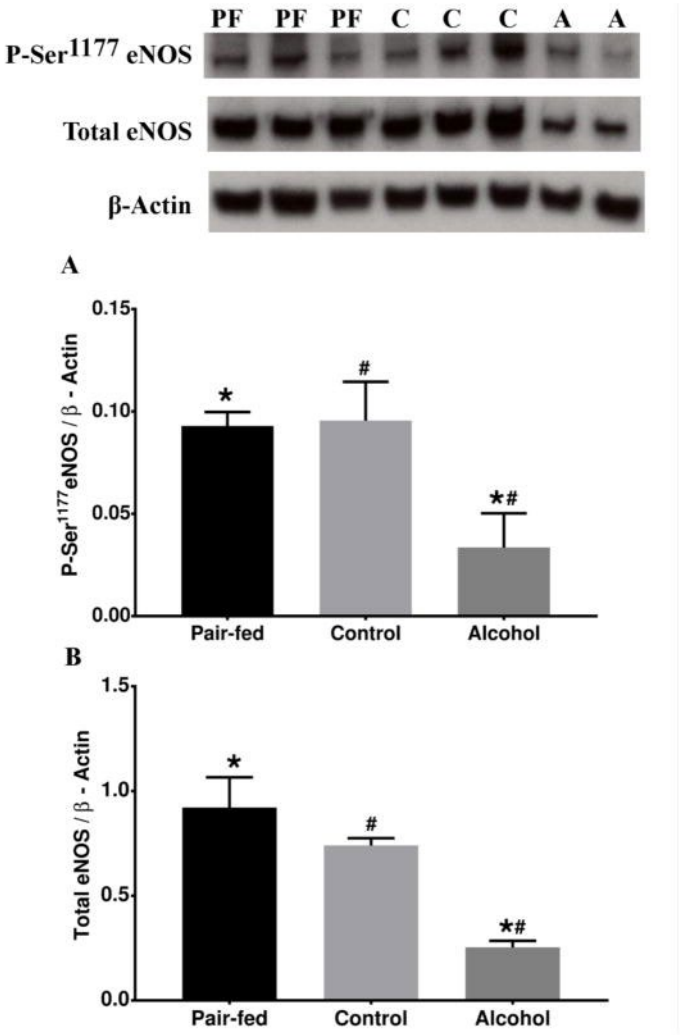


**Figure 8:** *Analysis of Total eNOS expression and phosphorylation level at Ser1177 eNOS by immunoblotting.* Uterine artery from GD 20 pregnant rats were homogenized and total eNOS protein expression and level of P-Ser1177 eNOS were analyzed by immunoblotting. Data are expressed as mean ±SEM and as fold of β-actin (loading control). Significance was established at priori at $P < 0.05$.

### 3.3.3.2. Immunohistochemical Analysis

Uterine artery sections isolated from pair-fed control and alcohol rats were probed with a P-Ser$^{1177}$eNOS antibody. Immunofluorescence imaging (Fig. 9) demonstrated that eNOS was detected exclusively in the uterine artery endothelial cells. The level of fluorescence showed major decreases in the levels of phosphorylation at the Ser$^{1177}$eNOS, thus complementing the immunoblotting analysis.


**Figure 9:** *Immunohistochemistry (IHC) analysis of P-Ser1177eNOS.* Immunofluorescence staining shows a localized expression of P-Ser1177eNOS in the endothelium and a major decrease of excitatory P-Ser1177eNOS expression in the uterine artery of the alcohol-administered rat compared to expression in the pair-fed control.

## 3.4. Discussion

This is the first study to demonstrate that *in vivo* binge alcohol exposure during pregnancy disrupts the endothelial NO-mediated vasodilation of the uterine artery. Collectively, 3 fundamental outcomes can be garnered from this study. During pregnancy, (i) alcohol significantly attenuates agonist-induced vascular reactivity in a rat uterine artery at physiological pressure of 90 mm Hg, (ii) alcohol disrupts uterine artery vasodilation via the eNOS pathway, and (iii) alcohol alters levels of excitatory posttranslational modification and protein expression of eNOS in the rat maternal uterine artery.

### 3.4.1. FAS Growth Restriction is Accompanied by Maternal Uterine Artery Dysfunction

In this study, pups from the alcohol-administered dams exhibited a significant decrease in fetal body weight when compared with pups from pair-fed control groups. Growth restriction is a hallmark phenotype of FAS, and our data align with previous studies performed using a similar exposure paradigm[1,32]. Furthermore, our study is the first to show that maternal uterine artery dysfunction accompanies FAS-related growth restriction. The current report corresponds with the previously reported observation that animal models of compromised pregnancies exhibit dysregulation of uterine vascular adaptations[33].

### 3.4.2. Gestational Alcohol Exposure Attenuates Vascular Reactivity

Chronic binge alcohol exposure during pregnancy attenuates uterine artery reactivity, a critical adaptation associated with pregnancy. During pregnancy, the maternal uterine vasculature undergoes extensive remodeling, characterized by a significant decrease in vascular resistance and a 30- to 50-fold increase in blood flow, ensuring adequate supply of oxygen and

nutrient delivery to the developing fetus[34]. These adaptations are in part associated with an increased endothelium-dependent vasodilation by 3 principal vasodilators, namely NO, $PGI_2$, and EDHF[35]. Herein, we examined endothelial-mediated vasodilation of the late-gestation primary uterine artery by inducing vasodilation via increasing concentrations of Ach, which acts on the muscarinic receptors on the endothelial cells, thereby releasing these key vasodilators. Our results indicated that chronic, binge-pattern, gestational alcohol exposure significantly diminishes endothelium-dependent uterine artery vasodilation.

Impaired uterine artery vasodilation during pregnancy may increase resistance to blood flow and consequently diminish nutrient and oxygen delivery to the fetus, potentially contributing to deleterious and lasting health and developmental consequences. Many studies suggest increased oxidative stress to play a role in alcohol-induced systemic vascular dysfunction, although so far only *in vitro* studies have been performed on the uterine artery, thus warranting future investigations in this area[8,36]. Our results align with prior studies, including studies on systemic (nonreproductive) vasculature, namely male rat tail artery, mesenteric artery, and aorta, and wire myography-based response in uterine artery[22], all of which concluded that endothelium-dependent relaxation is blunted in response to alcohol exposure[37–40].

### 3.4.3. Alcohol Disrupts the NO Pathway

Our data demonstrate that gestational alcohol disrupts the endothelial NO-mediated vasodilatory pathway in the primary uterine artery. In the absence of any inhibitors, alcohol decreased the maximal Ach-induced dilation by ~30%. Inhibition of the EDHF and $PGI_2$ pathways resulted in a significant, but most importantly, a further reduction in relaxation by ~46% in both treatment groups. This indicates that the contributions of EDHF and $PGI_2$ to

uterine artery vasodilation in both groups were of similar magnitude and not chiefly affected by alcohol. Thus, alcohol-induced dysregulation of Ach-induced vasodilation is mediated by impairment of NO component at least in the primary uterine artery. Interestingly, a similar loss of NO-mediated, endothelium-dependent relaxation was also observed in myometrial arteries of women with preeclampsia[41,42]. Other studies have demonstrated chronic alcohol exposure decreases arterial and plasma NO levels[43–45] and NO-mediated relaxation in rat systemic vasculature[46–48] aligning with our observation that alcohol impairs endothelium-mediated uterine artery vasodilation by affecting NO system.

To examine whether eNOS activity was affected in alcohol-fed rats, immunohistochemistry and immunoblotting experiments assessed maternal uterine artery excitatory Ser1177eNOS phosphorylation levels and eNOS expression. Decreased uterine vascular reactivity in alcohol-fed dams was concomitantly accompanied by a significant decrease in eNOS protein expression in uterine arteries compared to the untreated normal and pair-fed controls. Studies utilizing male rats have confirmed decreased renal[49] and aortic[44,50] eNOS levels following alcohol exposure. Prior studies utilizing isolated endothelial cell culture from uterine arteries of pregnant ewes[20] and human umbilical vein endothelial cells[21] ascertained that binge-like alcohol exposure decreased eNOS protein expression in these structures. Interestingly, immunoblot validation correspondingly revealed a significant decrease in the phosphorylation at the excitatory Ser$^{1177}$eNOS. Phosphorylation of the Akt-dependent Ser$^{1177}$eNOS is extensively associated with increased eNOS-mediated NO production in systemic vasculature[51].

Mutation at the Ser1177eNOS to mimic decreased phosphorylation attenuated NO release in various *in vitro* models[52,53]. Further, in vivo studies in rats have also found a direct relation between decreases in Ser$^{1177}$eNOS phosphorylation with decreased NO production[54–56].

In summary, this is the first study to demonstrate maternal binge alcohol consumption during pregnancy impairs uterine artery vascular reactivity, specifically impeding eNOS pathway-mediated vasodilation. Diminished vasodilation of the uterine artery can have deleterious effects on healthy fetal development. Substantial evidence robustly links low-birthweight infants with an increased risk of infant mortality and a heightened susceptibility for developing chronic diseases in later life, which experts recognize globally as the fetal origin hypothesis[57,58]. Thus, growth deficits incurred in response to alcohol exposure *in utero* may have lifelong health consequences that are potentially costly and can negatively influence both lifespan and quality of life. Although it cannot be said with certainty whether the decreased vasodilation would be directly responsible for the observed fetal growth restriction, this study provides a possible connection between the maternal uterine artery eNOS pathway and FASD pathogenesis. Future studies are warranted to ascertain specifically how these disruptions of uterine vascular adaptations to pregnancy may negatively impact fetal growth and development.

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# 4. REGIONAL DYSREGULATION OF TAURINE AND RELATED AMINO ACIDS IN THE FETAL RAT BRAIN FOLLOWING GESTATIONAL ALCOHOL EXPOSURE*

## 4.1. Introduction

Alcohol is an established teratogen responsible for a range of physical, physiological, neuroanatomical, and behavioral deficits collectively termed fetal alcohol spectrum disorders (FASD)[1,2]. In the United States, it is currently estimated that 1 in 10 pregnant women consumes alcohol, and 1 in 33 pregnant women reports binge drinking in the previous 30 days[3]. Alcohol consumption during pregnancy affects virtually every developing fetal organ system, of which the most-studied target is the developing brain. The deficits reported include whole and regional brain volume reductions, cortical dysmorphology, neuronal depletion, disruption of neuronal differentiation and migration, and neurobehavioral deficits including learning, memory, and attention impairments[4,5]. Since 1968, an extensive body of work has cataloged the effect of alcohol on the developing brain[5–8]. However, delineation of candidate mechanisms underlying alcohol-induced neurological deficits remains indeterminate, attributed in part to the complexity of alcohol's cellular targets and pharmacokinetics, as well as varying temporal and regional vulnerability observed in the brain throughout gestation[9–11].

Gestational alcohol exposure significantly impairs AA bioavailability in both the mother and the developing fetus. In rodent models, maternal plasma threonine, serine, glutamine, glycine, alanine, and methionine are reduced following an acute alcohol exposure[12], and plasma

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proline diminishes following chronic exposure[13]. Our group demonstrated maternal and fetal plasma AA dysregulation including glutamine and glutamate in response to alcohol[14,15]. In addition, impairment of maternal uterine artery blood flow and placental uptake of AAs are observed following maternal alcohol consumption, and these factors all critically regulate fetal AA bioavailability[16]. Maternal alcohol consumption and AA disruption are directly associated with intrauterine growth restriction (IUGR), which may potentiate the risk for a myriad of adult-onset diseases[17–19]. Despite this evidence regarding the effects of alcohol on AA homeostasis, to the best of our knowledge, no information is available on the AA profile in the alcohol-sensitive developing brain, regionally or holistically, in FASD.

In addition to their basic role as protein building blocks, AAs also function as precursors for neurotransmitters, nucleotides, sphingolipids, polyamines, as donors for nitric oxide[20], and as potent antioxidants, regulators of hormone secretion, and signaling modulators[20]. AAs are the most abundant neurotransmitters in the brain, and act as neurotrophic factors, playing major roles in synaptogenesis, neuronal proliferation, and migration[21]. Outside the FASD field, AA analysis in developing brain regions has led to significant advances in understanding the mechanistic pathways leading to neuropathology.

The purpose of this study was to explore the effect of alcohol on region-specific patterns of AA abundance. We chose to explore the cerebral cortex, the hippocampus, and the cerebellum, as these regions have previously shown high alcohol-induced vulnerability, manifesting as a myriad of developmental deficits[6,10,22]. We hypothesized that gestational alcohol exposure will alter brain AA concentrations, causing disruption to key AAs associated with neuropathogenesis, and that these alterations will be region-specific.

## 4.2. Materials and Methods

### 4.2.1. Animals

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996), with approval by the Animal Care and Use Committee at Texas A&M University. Timed pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA), and were housed in a temperature-controlled room (23 °C) with a 12:12-h light/dark cycle. Rats were assigned to a pair-fed control group (n = 6) or an alcohol treatment group (n = 6) on GD 4. The alcohol animals were acclimatized via a once-daily orogastric gavage of a 4.5 g/kg (22.5% wt/v) alcohol dose from GD 5–10 and progressed to a 6 g/kg alcohol dose from GD 11–20 (28.5% wt/v). The pair-fed control rats were isocalorically matched to alcohol rats by daily dosing with a gavage of maltose dextrin to account for calories from alcohol. The exposure regimen utilized in this study was based on both reported binge alcohol consumption patterns in pregnant women and binge exposure patterns implemented across FASD animal models[23–28]. All rats were weighed prior to the start of the study, and each treatment animal was yoked with a control animal of similar weight throughout the duration of the study. Food intake in both groups was measured daily and the amount of diet consumed by the pair-fed animals was matched with the alcohol-fed animals. There was no significant maternal weight difference between the groups on GD 21. Animals were sacrificed on GD 21, one day after the last alcohol exposure. Litter size between treatment groups was not different ($p = 0.77$).

### 4.2.2. Fetal Brain Region Isolation

Fetal brain tissue was collected from an equal number of male and female offspring within each treatment group. A single fetal brain per dam was utilized for sample analysis. Samples were serially washed in cold phosphate-buffered saline (PBS), meninges were removed, and the bilateral hippocampi, cerebellum, and whole cerebral cortex were micro-dissected in ice-cold HEPES buffer. Individual samples were then flash-frozen and stored at −80 °C.

### 4.2.3. Sample Preparation

Fetal brain tissue was weighed, subsequently acidified with 50 µl of 1.5-mM $HClO_4$, homogenized in 925-µL $H_2O$, and then neutralized with 25 µl of 2-mM $K_2CO_3$[29]. The supernatant fluid was used for AA analysis by HPLC, as described previously [30]. Concentrations of AAs in samples were quantified based on authentic standards from Sigma Chemicals (St. Louis, MO, USA), using the Waters Millenium-32 workstation[31,32].

### 4.2.4. Statistical Analysis

The concentrations of AAs in the cortex, cerebellum, and hippocampus of control and alcohol animals were analyzed by Student's t test. Statistical significance was established *a priori* at $p < 0.05$.

## 4.3. Results

There was no significant difference in the maternal weight between the pair-fed control and alcohol-fed dams on GD 20 (pair-fed control, 309 ± 8 g; alcohol, 308 ± 15 g). Fetal weight was significantly decreased in the alcohol group (2.12 ± 0.11 g), compared with that in the pair-

fed control (2.53 ± 0.06 g). Concentrations of 22 AAs within each of the three brain regions of interest (cerebral cortex, hippocampus, and cerebellum) for both control and alcohol animals are summarized in Table 3 (Appendix A).

The most notable changes in AA concentrations were observed in the fetal cerebellum and hippocampus, two structures established as exquisitely sensitive to prenatal alcohol exposure[10].

### 4.3.1. Fetal Cerebral Cortical AA Dysregulation

In the cerebral cortex (Fig. 11), taurine was the most abundant AA, followed by glutamate, glutamine, alanine, and serine. Of the 22 AAs analyzed, concentrations of 8 AAs were significantly different ($p < 0.05$) between the pair-fed control and alcohol groups. Cortical isoleucine ($p = 0.0093$), serine ($p = 0.0416$), valine ($p = 0.0205$), leucine ($p = 0.0170$), and ornithine ($p = 0.0174$) concentrations significantly increased in the cerebral cortex of gestational alcohol-exposed offspring compared with those in pair-fed controls. In contrast, glutamine ($p = 0.0057$), taurine ($p = 0.0113$), and tryptophan ($p = 0.0078$) were significantly decreased in the alcohol group compared to pair-fed controls. Of the three structures analyzed, the cerebral cortex had the fewest number of significantly different AA concentrations.

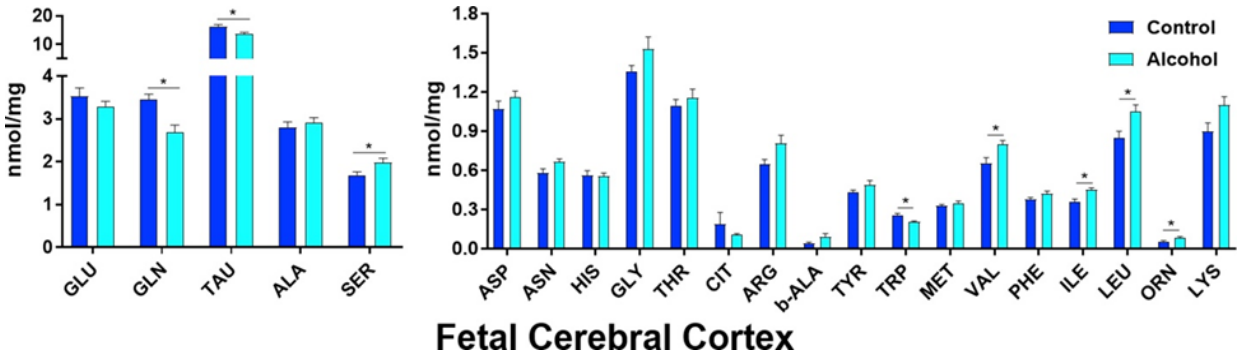


**Figure 10:** *Effect of chronic gestational binge alcohol exposure on fetal cortical amino acid concentrations (nmol/mg*). Left: Most abundant fetal brain amino acids: Levels of glutamine (↓), taurine (↓), and serine (↑) were significantly different within cortical tissue in the alcohol group compared with those in the pair-fed control group. Right: Less abundant fetal brain amino acids: Isoleucine (↑), leucine (↑), valine (↑), ornithine (↑), and tryptophan (↓) were significantly different within cortical tissue in the alcohol group compared with those in the pair-fed control group. *Indicates statistically significant difference in amino acid concentration, $p < 0.05$.

### 4.3.2. Fetal Cerebellar AA Dysregulation

Within the cerebellum (Fig. 12), taurine was the most abundant AA, followed by glutamate, glutamine, and alanine in both treatment groups. Interestingly, the fifth most abundant AA was glycine in the control group and serine in the alcohol group. Of the 22 AAs analyzed, 20 (~91%) AA concentrations were significantly different ($p < 0.05$) between the pair-fed control and alcohol groups. All 20 of these AA concentrations were increased in alcohol-exposed cerebella compared with those from pair-fed control animals. These AAs were alanine ($p = 0.000069$), arginine ($p = 0.0000096$), asparagine ($p = 0.0000099$), aspartate ($p = 0.00026$), glutamine ($p = 0.0026$), glutamate ($p = 0.0013$), glycine ($p = 0.000027$), histidine ($p = 0.000042$), isoleucine ($p = 0.0000061$), leucine ($p = 0.0000061$), lysine ($p = 0.000017$), methionine ($p = 0.000018$), ornithine ($p = 0.0000033$), phenylalanine ($p = 0.0000068$), serine ($p = 0.0000059$), taurine ($p = 0.034$), threonine ($p = 0.0000046$), tryptophan ($p = 0.00071$), tyrosine ($p =$

0.0000023), and valine (p = 0.0000056). The cerebellum showed more alteration than the cerebral cortex, but less than that in the hippocampus.

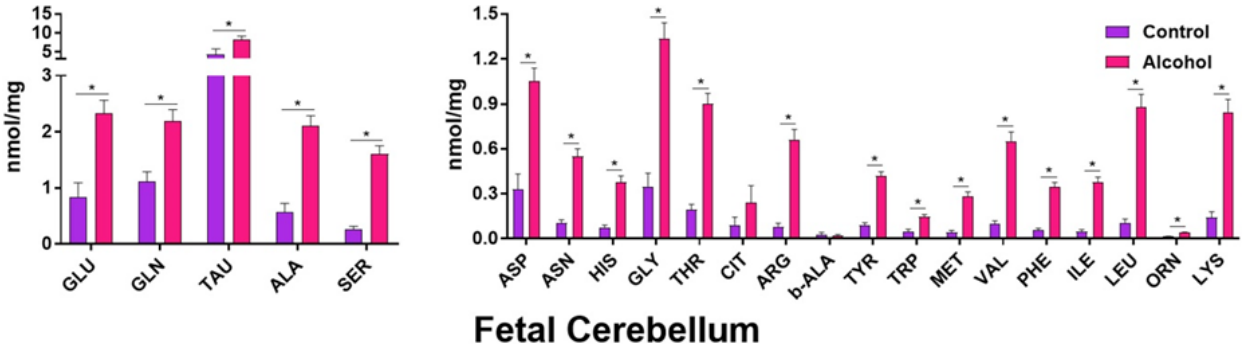


**Figure 11:** *Effect of chronic gestational binge alcohol exposure on fetal cerebellar amino acid concentrations (nmol/mg)*. Left: Most abundant fetal brain amino acids: Glutamate, glutamine, taurine, alanine, and serine levels significantly increased (↑) in cerebellar tissue in the alcohol group compared with those in the pair-fed control group. Right: Less abundant fetal brain amino acids: Asparagine, aspartate, histidine, glycine, threonine, arginine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine, ornithine, and lysine levels significantly increased (↑) in cerebellar tissue in response to gestational alcohol exposure. *Indicates statistically significant difference in amino acid concentration, $p < 0.05$.

### 4.3.3. Fetal Hippocampal AA Dysregulation

Taurine was the most abundant AA in the hippocampus (Fig. 13), followed by glutamate, glutamine, alanine, and serine in both treatment groups. Representative chromatogram traces are shown from (A) standards, (B) pair-fed control, and (C) alcohol treatment groups (Fig. 14). All 22 AAs analyzed were significantly different between treatment groups ($p < 0.01$). Every

hippocampal AA concentration increased in the alcohol group compared with those in the pair-fed control group. Of the three structures analyzed, the hippocampus had the highest percentage of significantly different AA concentrations between treatment groups. These AAs were alanine ($p = 0.00011$), arginine ($p = 0.0000043$), asparagine ($p = 0.000013$), aspartate ($p = 0.000033$), β-alanine ($p = 0.0015$), citrulline ($p = 0.0012$), glutamine ($p = 0.00052$), glutamate ($p = 0.00035$), histidine ($p = 0.000011$), isoleucine ($p = 0.0000039$), leucine ($p = 0.0000047$), lysine ($p = 0.000010$), methionine ($p = 0.000013$), ornithine ($p = 0.0012$), phenylalanine ($p = 0.0000091$), serine ($p = 0.0000074$), taurine ($p = 0.0038$), threonine ($p = 0.000022$), tryptophan ($p = 0.0052$), tyrosine ($p = 0.000019$), and valine ($p = 0.0000071$).

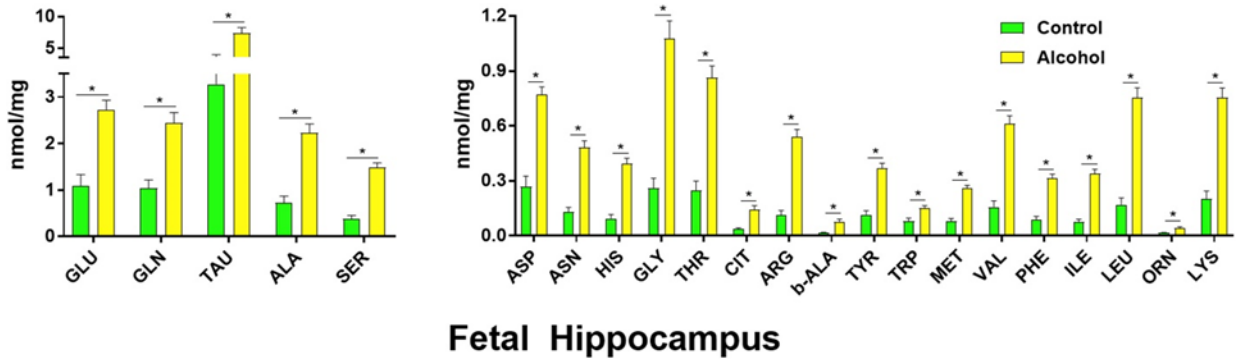


**Figure 12:** *Effect of chronic gestational binge alcohol exposure on fetal hippocampal amino acid concentrations (nmol/mg)*. Left: Most abundant fetal brain amino acids: Glutamate, glutamine, taurine, alanine, and serine levels significantly increased (↑) in hippocampal tissue in the alcohol group compared with those in the pair-fed control group. Right: Less abundant fetal brain amino acids: Asparagine, aspartate, histidine, glycine, threonine, citrulline, arginine, β-alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, leucine, ornithine, and lysine levels significantly increased (↑) in hippocampal tissue in the alcohol group compared with those in the pair-fed control group. *Indicates statistically significant difference in amino acid concentration, $p < 0.05$.

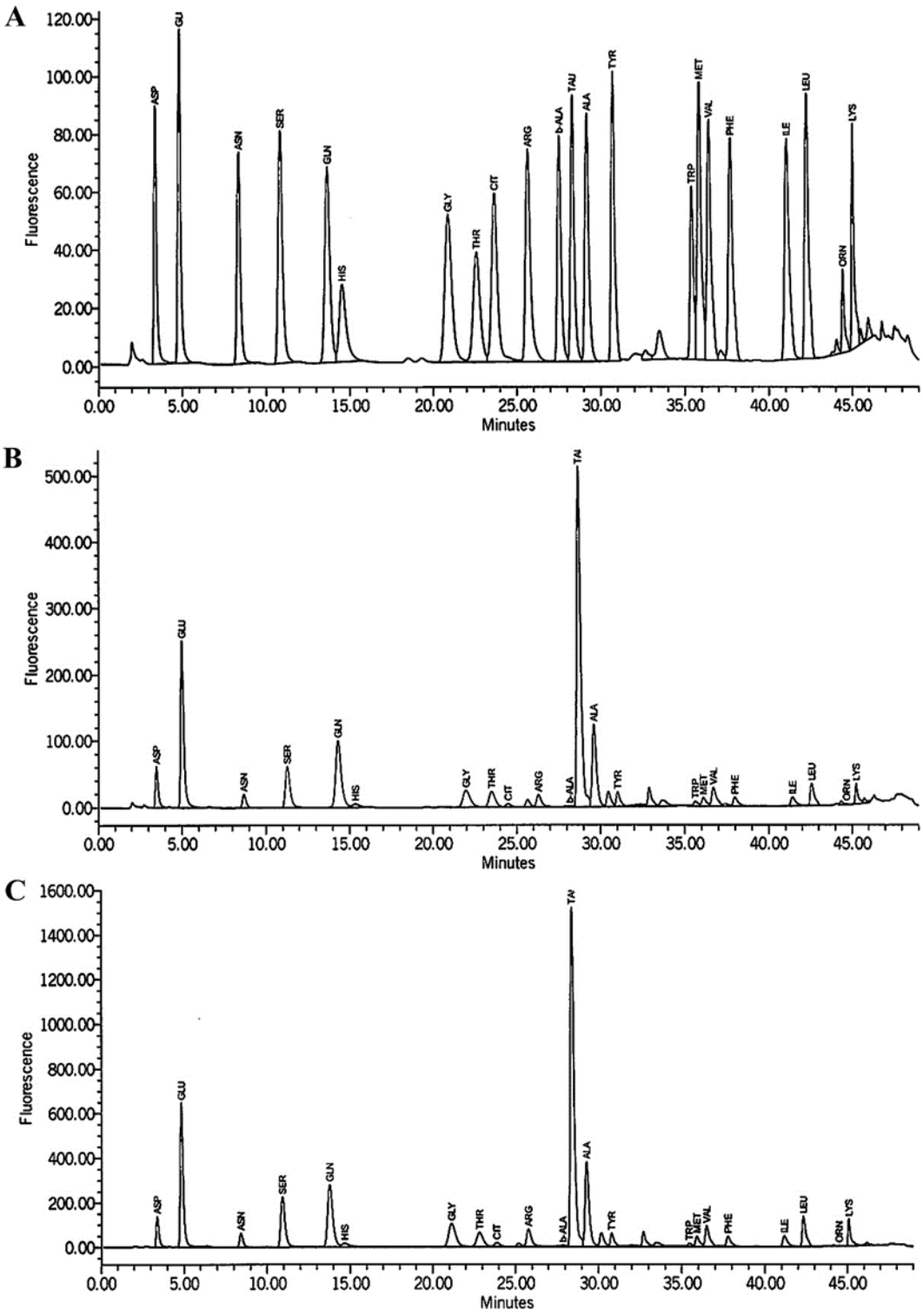


**Figure 13:** *Example chromatogram traces from fetal hippocampal tissue.* Representative chromatogram traces are shown from (A) standards, (B) pair-fed control, and (C) alcohol treatment groups

## 4.4. Discussion

To our knowledge, this is the first study distinguishing dynamic, region-specific, alterations of AA concentrations in the developing fetal brain in response to gestational alcohol exposure. The cerebral cortex showed the fewest altered AA concentrations, in contrast to the cerebellum and hippocampus, which exhibited dramatic AA dysregulation. Additionally, our data demonstrate that prenatal alcohol exposure regionally increases excitatory amino acids in the fetal brain, and also increases taurine levels in these same regions. These AA imbalances may provide insight into both alcohol-mediated neuropathogenesis and the brain's compensatory neuroprotective response to prenatal alcohol exposure.

### 4.4.1. Alcohol Significantly Increases Hippocampal and Cerebellar Excitatory AAs

In alcohol-exposed offspring, considerable increases in glutamate were detected in the cerebellum (177.40%) and hippocampus (149.00%). Aspartate also increased in the cerebellum (218.54%) and hippocampus (186.73%) in the alcohol group compared with pair-fed controls. Excitatory AAs, which include glutamate and aspartate, act on voltage-gated channels in plasma membranes throughout cell populations in the brain[33]. Studies show increases of these AAs in the developing brain in response to traumatic brain injury[34], hypoxia-ischemia[35], and maternal stress[36]. Excitatory AA disruption has also been observed previously in offspring brain regions following intrauterine inflammation[37]. In the developing brain, acute increases in concentrations of excitatory neurotransmitters lead to acute neurotoxicity, as well as impairment in neurotransmitter programming, receptor expression, neuronal migration, and synapse maturation, and have been linked with numerous long-term behavioral deficits and psychiatric illnesses[21]. Our data indicate that alcohol induces significant regional increases in glutamate and aspartate

and increases of these AAs may be responsible for neuronal death observed in the hippocampus and cerebellum following alcohol exposure, two regions with well-documented acute alcohol vulnerability. Interestingly, branched-chain AAs, valine, leucine, and isoleucine exhibited significant increases in the cerebellum and hippocampus, respectively, following alcohol exposure. Together, these branched-chain AAs function as primary nitrogen donors in glutamate synthesis, and thus may be integral in the marked increase we observed in the excitatory AA glutamate[38].

### 4.4.2. Alcohol Regionally Alters Taurine Levels, the Most Abundant AA in the Developing Brain

Our data illustrate that gestational alcohol regionally alters taurine distribution in the cerebral cortex (−16.31%), cerebellum (+88.85%), and hippocampus (+126.71%). Alcohol also increased methionine, a direct taurine precursor, in both of these structures (cerebellum: 550.88%; hippocampus: 230.95%). Taurine is the most abundant AA in the developing brain, exhibiting region-specific abundance and significantly higher levels in the developing versus mature brain[39]. By region, taurine concentration is highest in the olfactory bulbs (an area of sustained neurogenesis), followed by the cerebellum and then cerebral cortex. By cell type, taurine concentration is highest in the cerebellar Purkinje cells, an established target exquisitely vulnerable to prenatal alcohol exposure. Taurine is the highest free AA in milk, further suggesting a role in neuronal maturation. Across species, taurine is critically implicated in brain development and in maintaining neuronal homeostasis by acting as a model osmoregulator and intracellular calcium modulator[39,40]. Taurine deficiency during development correlates with impaired neuronal migration, proliferation, and organization, yet the mechanisms of taurine

requirements for nervous system maturation are not yet fully understood[39]. Taurine disruption in the developing brain correlates with impaired sensory integration and cortical processing, yet not with somatic growth impairments[41,42]. This phenotype is a hallmark for many children affected with FASDs, and our working hypothesis is that taurine is a key mechanistic component involved in neuropathology underlying behavioral deficits of FASD.

### 4.4.3. Our Working Model

Interestingly, taurine and glutamate are linked in a unique cell-signaling pathway within the brain (Fig. 15). Previous studies show that excessive glutamate release disrupts intracellular calcium homeostasis, increasing intracellular osmolality[40,43]. In response, taurine levels increase to regulate these increases back within homeostatic levels, actively preventing neuronal death induced via excitatory neurotoxicity[44,45]. Based on our findings, we hypothesize that in select developing brain regions, gestational alcohol induces excessive glutamate release, potentially leading to intracellular hypertonicity; in response, taurine increases downstream in an effort to normalize glutamate-induced excitatory neurotoxicity. We further propose taurine may be acting as a compelling neuroprotectant in response to this exposure. We conjecture that AA imbalances observed in this study are critically implicated in pathological and compensatory processes occurring in the brain in response to gestational alcohol exposure.

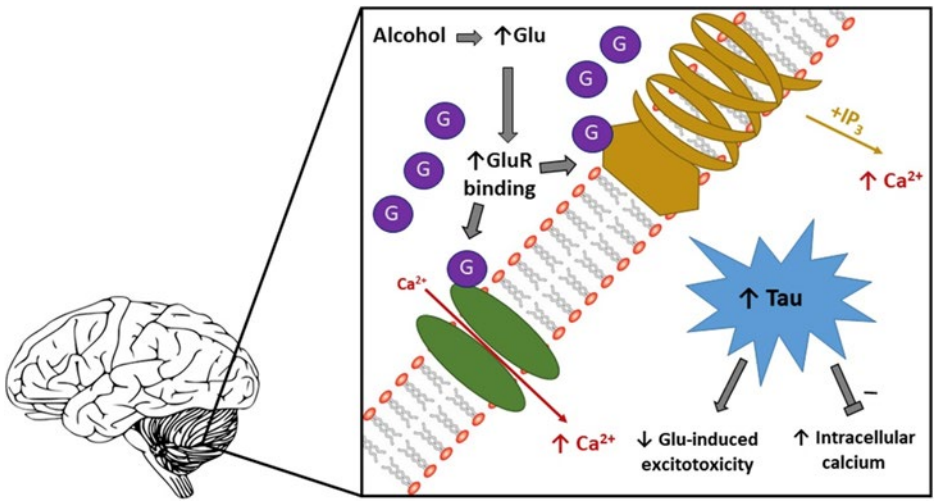


**Figure 14:** *Our working hypothesis.* Glutamate and taurine increase in the fetal cerebellum and hippocampus following a chronic, binge, gestational alcohol-exposure paradigm. We theorize that this alcohol exposure increases glutamate and subsequent glutamate receptor (NMDA and/or metabotropic) binding in these structures, inducing calcium influx, in turn stimulating excitatory neurotoxicity leading to cell death, and potentially accounting for previously observed neuronal impairment following similar exposure paradigms. We conjecture that taurine, an ideal osmolyte, increases in response to glutamate-induced calcium influx, counteracting osmotic disruption and offsetting alcohol-mediated damage within these distinctly vulnerable structures.

### 4.4.4. Study Limitations

It should be noted that in our chronic binge alcohol paradigm, withdrawal might play an influential role in fetal brain AA homeostasis. Our results may indicate a direct alcohol effect, alcohol withdrawal consequences, or a combined effect of both. Previous studies have ascertained that key brain excitatory amino acids increase in response to alcohol withdrawal[46–49], though this is the first study analyzing an AA profile, including excitatory AAs and their precursors, following a chronic binge gestational exposure. An additional consideration worth noting is that the AA disruption observed in this study may be a reflection of alcohol-induced

cellular impairment rather than alcohol teratogenicity. For instance, observed AA alterations may be an accumulative effect, reflective of alcohol-induced impairment of cellular processes, which could in turn affect AA metabolism and utilization, differentiation, and/or migration[50]. Future studies of other brain regions and additional developmental time-points are warranted to understand how gestational alcohol exposure affects global AA homeostasis in the developing brain and to discern further mechanistic insights.

### 4.4.5. Perspectives

In the last half century, vast progress has been made discerning targets of gestational alcohol exposure, yet limited knowledge persists for the mechanisms underlying alcohol-mediated developmental pathology. Understanding foundational building blocks for pathogenesis, such as alcohol-induced disruption of AA homeostasis essential for development, is key for mechanistic discernment. Collectively, our data demonstrate that alcohol-induced dysregulation of AA concentrations in the developing brain are region-specific. Future studies are warranted in identifying mechanisms underlying the observed AA dysregulation and whether these mechanisms are region-specific. Further discernment of these mechanisms would advance the FASD field by providing pivotal insight into alcohol's action on specific regions of the fetal brain, as well as by identifying strategic sites for therapeutic intervention.

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# 5. HIPPOCAMPAL TRANSCRIPTOME REVEALS NOVEL TARGETS OF FASD PATHOGENESIS*

## 5.1. Introduction

Fetal alcohol spectrum disorders (FASD) collectively describe an array of physical abnormalities, central nervous system disruptions, and cognitive and behavioral deficits induced by prenatal alcohol exposure[1,2]. In the United States, more than 30% of pregnancies are estimated to be affected by prenatal alcohol exposure[3], and one in 10 pregnant women report alcohol consumption in the past 30 days[4]. A recent study estimates that FASD prevalence in the U.S. populations may range from 3% up to 9%[5]. A myriad of factors influences phenotypic severity within FASD, including timing, dose, and duration of exposure, as well as maternal nutrition, genetic susceptibility of both the mother and fetus, and parental history of substance use disorder[6–9]. These variables, coupled with the fact that *in utero* alcohol exposure impairs nearly every developing organ system, attribute to the wide-ranging variation in the presentation and severity of FASD phenotypes among affected individuals[10–12]. Birth defects resulting from prenatal alcohol exposure are persistent and lifelong, with profound socioeconomic consequences[13]; currently no approved pharmacologic therapy exists[14]. Targets of prenatal alcohol exposure and its pharmacokinetics are complex in nature, and thus to date, the molecular mechanisms underlying FASD pathogenesis remain insufficiently understood[15].

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The fetal brain is one of the most well-studied targets of gestational alcohol exposure. Human and animal model studies have implicated the developing hippocampus, a structure associated with learning and memory function, as exquisitely vulnerable to alcohol-induced developmental damage[16,17]. In animal models, gestational alcohol-induced alterations to hippocampal synaptic plasticity have been extensively studied[18,19], as well as alcohol-induced alterations to hippocampal synaptic activity[20] and regional and cellular morphology[21–23]. In humans, prenatal alcohol exposure produces asymmetrical reduction in hippocampal volume, impaired spatial recall, delayed reproduction of a spatial figure, impaired place learning, delayed recognition, and verbal learning tasks relative to controls[24–26].

A limited number of FASD animal model studies have reported alterations in the hippocampal transcriptome using DNA microarray analysis[27–29]. One study reported that developmental alcohol dysregulates several genes implicated in the nervous system development (Nova1, Ntng1, Neurog2, and Fexfs)[29], and another reports that alcohol alters hippocampal gene expression, DNA methylation, and histone methylation in free radical scavenging networks in offspring 70 days after birth[27]. FASD studies have also shown altered hippocampal DNA methylation and gene expression on postnatal day (PND) 28 corresponding with asymmetrical hippocampal volume on PND 60 in offspring exposed to alcohol during early neurulation (GDs 0.5–8)[30], and that alcohol exposure on GDs 8–21 dysregulates several candidate genes (Gabrb3, Ube3a, Mecp2, and SLC25a12) that overlap with autism spectrum disorders and concurrently produces adverse hippocampal learning outcomes in adult offspring[31,32].

These studies largely utilize microarrays to assess gene expression in mature offspring, a time when hippocampal-based learning outcomes can be effectively assessed. Our study is unique as it is the first to utilize high-throughput next-generation (next-gen) RNA deep-

sequencing (RNA-seq) to examine a more thorough, dynamic range of transcriptome-wide effects of chronic prenatal binge alcohol exposure on the developing hippocampus. Alcohol-induced dysregulation to the hippocampal transcriptome during pregnancy could substantially impair hippocampal development and associated adverse consequences may impair juvenile learning outcomes that could persist into adulthood. It is essential to understand the fully alcohol-induced hippocampal transcriptome dysregulation early in life so that targeted intervention strategies may be effectively applied as soon as possible. However, microarray analysis is restricted in its ability to detect differentially expressed genes due to factors such as high background levels caused by cross-hybridization and signal saturation, and also lacks sensitivity for genes with very high or low expression levels[33]. By utilizing next-gen RNA-Seq as a strategic means for investigating multi-mechanistic actions of alcohol on holistic gene expression of target organ structures, a much larger dynamic range of differentially expressed hippocampal genes can be detected[33]. Since alcohol has been shown to affect various aspects of fetal hippocampal development[34–36], it is imperative to further discern how gestational alcohol exposure alters this structure at the level of the transcriptome so that we may understand mechanisms underlying neuropathogenesis and develop appropriately targeted intervention strategies.

Our model of FASD has previously shown distinct dysregulation of amino acid homeostasis and the protein signature in the fetal hippocampus[37]. The purpose of the study was to discern the alterations of fetal hippocampal gene expression and their associated pathways in response to maternal alcohol exposure. We hypothesized that chronic gestational alcohol exposure alters fetal hippocampal gene expression and their related global canonical pathways.

## 5.2. Materials and Methods

### 5.2.1. Animals

All experimental procedures were in accordance with the National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at Texas A&M University. Timed-pregnant Sprague–Dawley rats were purchased from Charles River (Wilmington, MA), and were housed in a temperature-controlled room (23°C) with a 12:12-hr light–dark cycle. Rats were assigned to a pair-fed control (PF) group (n = 6 dams) or an alcohol (ALC) treatment group (n = 6 dams) on GD 4. The ALC-treated animals acclimatized via a once daily orogastric gavage of a 4.5 g/kg (22.5% wt/v, peak BAC, 216 mg/dl) alcohol dose from GDs 5–10 and progressed to a 6 g/kg dose (28.5% wt/v, peak BAC, 289 mg/dl)[37] from GDs 11–20. The PF animals were isocalorically matched to the ALCs by daily dosing with a gavage of maltose dextrin to account for calories derived from alcohol. The exposure regimen utilized in this study is based on both reported binge alcohol consumption patterns in pregnant women and binge exposure patterns implemented across FASD animal models[7,38–42]. All rats were weighed prior to the start of the study, and each treatment animal was yoked with a control animal of similar weight throughout the duration of the study. Feed intake in both groups was measured daily and the amount of diet consumed by the ALC animals was matched to the diet administered to PF animals. There was no significant maternal weight difference between treatment groups. Animals were sacrificed on GD 21, one day after the last alcohol exposure.

### 5.2.2. Fetal Hippocampal Isolation

Fetal brain tissue was collected from an equal number of male and female offspring within each treatment group. Brains were extracted under a dissection microscope via craniotomy and were serially washed in cold phosphate buffered saline (PBS), meninges were removed, and bilateral hippocampi were microdissected in ice-cold HEPES buffer. Individual samples were then flash frozen and stored at −80 ◦C until analyses. One pair of male or female hippocampi from each dam was utilized for analysis.

### 5.2.3. Sample Preparation

Each tissue sample was homogenized in TRIzol® Reagent and total RNA was isolated according to manufacturer's protocol (Invitrogen; Carlsbad, CA). Prior to analysis, RNA quality was assessed using an Agilent TapeStation RNA assay. Whole-genome RNA transcripts were quantified via Qubit Fluorometric assay and subsequently all samples were normalized to an equivalent starting concentration. Sequencing libraries were prepared using the TruSeq Stranded mRNA Library Prep kit (Illumina; San Diego, CA). Each sample was uniquely indexed (barcoded) to allow for pooling of all samples in a single sequencing run. Library size and quality were then assessed with an Agilent TapeStation D1000 DNA assay. Samples were normalized to ~4nM and pooled equally. Sequencing was performed on an Illumina NextSeq 500 running with a 75 cycle, single-end sequencing run.

### 5.2.4. Bioinformatics

Raw RNA-sequence data were analyzed to identify significant differences in gene expression between the PF and ALC treatment groups, sex-dependent expression differences

between these treatment groups, and the global biological pathways associated with disruption of these hippocampal genes. A total of approximately 142 million reads were evaluated and trimmed of all adapter sequences and low quality bases using Trimmomatic read trimmer 43. Using Trimmomatic and the corresponding adapter sequences file for Illumina, reads were scanned with a sliding window of 5, cutting when the average quality per base drops below 20, then trimming reads at the beginning and end if base quality drops below 20, and finally dropping reads if the read length is less than 50. This resulted in 131 million filtered reads (approximately 92%), of which a total of 128 million filtered reads (approximately 97%) were mapped to the Rattus norvegicus (rn5) genome assembly. Read mapping for our samples was performed using HISAT genomic analysis software platform version 2.0.5 44. Transcript-wise counts were generated using the featureCounts tool from the SUBREAD high-performance read alignment package 45. Differential gene expression tests were then performed using DESeq2 software following the guidelines recommended by Love and colleagues 46. Heat map and volcano plots were generated from this processed data using the R programming language. The resulting gene expression values for genes that met statistical significance criteria were uploaded to INGENUITY® Pathways (QIAGEN, Venlo, Netherlands; Application Build 261899, Content Version 18030641) for biological pathway analysis. A core analysis was used to identify top canonical pathways effected by the alcohol treatment. Filters utilized for this analysis include species, confidence, mutation, and molecule type.

### 5.2.5. Statistical Analyses

Raw read counts for each gene in each hippocampal sample were utilized as input into DESeq2, which modeled the read counts as following a negative binomial distribution, with a

mean representing the read concentration per gene. This mean was scaled by a normalization factor (median-of-ratios) to account for differences in sequencing depth between samples. During independent filtering, DESeq2 used the average expression strength of each gene, across all samples as its filter criteria, and omitted all genes with mean normalized counts below a filtering threshold from multiple testing adjustments. The genes that satisfied $-2 > \log2(\text{fold change}) > 2$ and $p < 0.05$ were deemed differentially expressed. Median-of-ratios for each gene was determined as a raw count of the gene divided by the row-wise geometric mean to yield a ratio and a median of ratios for all genes in each sample, thus producing a normalization factor for the sample. After normalized counts were calculated for each gene in each sample, a generalized linear model (GLM) with a logarithmic link was fit in order to test for treatment effects (alcohol vs. control) and conditional effects (sex), which returned the coefficients indicating overall expression strength of a gene and log2(fold change) between the treatment groups. After GLMs were fit for each gene, DESeq2 utilized a Wald test for significance (to test the null hypothesis that the logarithmic fold change between the treatment and control group is exactly zero for a given gene's expression), and the resulting Wald test p values of a subset of genes that pass independent filtering were adjusted for multiple testing using the Benjamini–Hochberg procedure. During independent filtering, DESeq2 used the average expression strength of each gene, across all samples, as its filter criteria, and omitted all genes with mean normalized counts below a filtering threshold from multiple testing adjustments. By default, DESeq2 chose a threshold that maximized the number of genes found at a user-specified target false discovery rate (FDR; 0.05). Gene sets that satisfied log2 (fold change) $\geq 2.0$ and an FDR adjusted P-value $<0.05$ were considered differentially expressed.

### 5.3. Results

High-throughput RNA deep-sequencing analysis identified 13,388 hippocampal genes, of which 76 showed significant dysregulation following chronic binge gestational alcohol exposure ($p < 0.05$; log2(fold change) $\geq 2.0$). Of these dysregulated genes, 37 exhibited downregulation and 39 expressed upregulation. A heat map illustrates these alterations (Figure 15); expression values based on Pearson correlation values determined the hierarchical clustering structure. Within this group of dysregulated genes, a subset of 49 genes showed sex-dependent expression differences ($p < 0.05$; log2(fold change) $\geq 2.0$), with 23 genes in alcohol-exposed females and 26 genes in alcohol-exposed males showing expression differences when compared to respective PF offspring. Two genes, ATP synthase F1 subunit (Atp5f1) and Smad nuclear interacting protein 1 (Snip1) exhibited significant dysregulation in both alcohol-exposed females and males. Interestingly, Atp5f1 expression increased in ALC females but decreased in ALC males. Snip1 expression decreased in ALC female and male offspring.

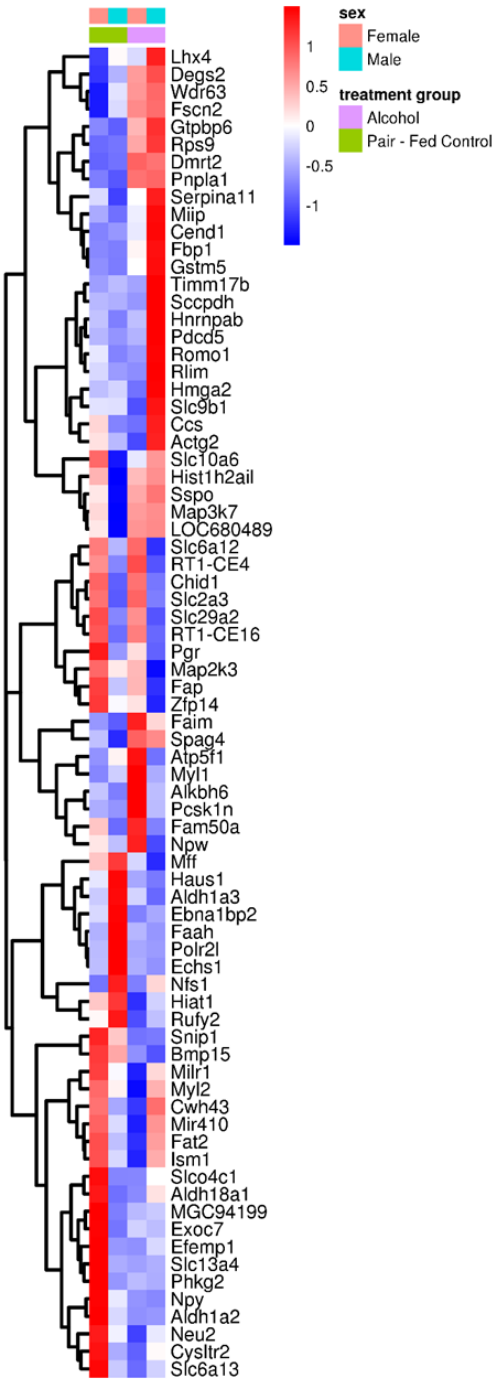


**Figure 15:** *Heat map of RNA-Seq transcriptome analysis of significantly altered hippocampal genes following our chronic binge prenatal alcohol paradigm*. Heat map representation of 76 differentially expressed genes in the fetal hippocampus between pair-fed Control and alcohol treatment groups, with 37 genes exhibiting downregulation and 39 genes exhibiting upregulation. Map was constructed from the normalized and log-transformed expression values and subtracted from the row means for each treatment group ($p < 0.05$, and log2 (fold change) $\geq 2.0$)

Among the 26 hippocampal genes exhibiting expression changes in ALC females, nine displayed upregulation and 16 displayed downregulation (Figure 16). Two downregulated genes, aldehyde dehydrogenase 18 family, member A1 (Aldh18a1, ↓) and microRNA 410 (Mir410, ↓), have gene-chemical interactions with choline, an essential nutrient and methyl donor which has been shown to be dysregulated following developmental alcohol exposure and is critically implicated in hippocampal-based learning tasks[41,47]. Two other downregulated genes, myosin light chain 2 (Myl2, ↓) and phosphorylase kinase catalytic subunit gamma 2 (Phkg2, ↓), have gene-chemical interactions with ethanol. Proprotein convertase subtilisin/kexin type 1 inhibitor (Pcsk1, ↑) has previously been identified as involved in brain development, and is also implicated in neuroendocrine signaling[48]. Other genes of interest include neuraminidase 2 (Neu2, ↓), for which response to ethanol is a biological process and solute carrier family 6 member 13 (Slc6a13, ↓) which is involved in neurotransmitter transport and binding.

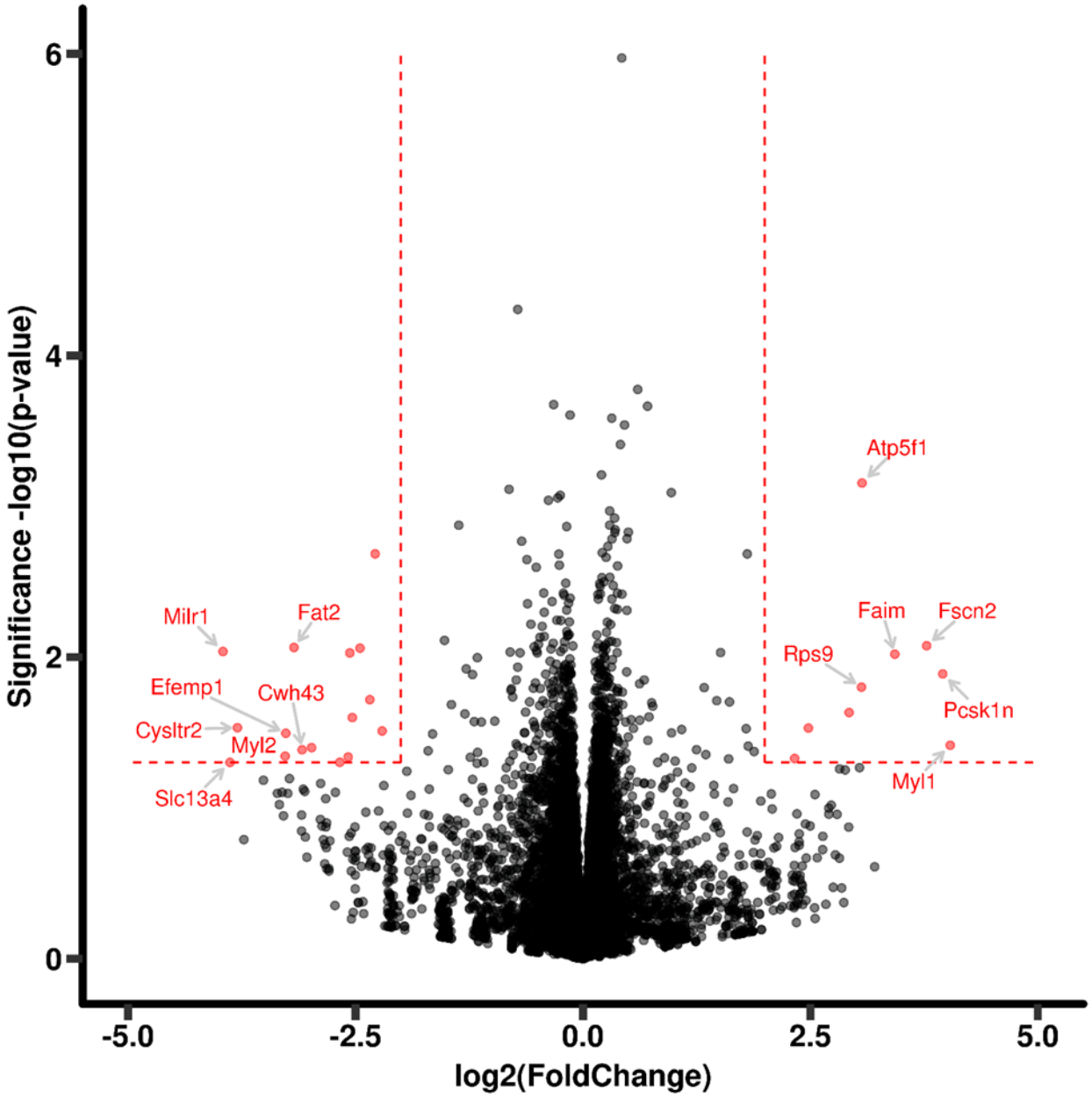


**Figure 16:** *Volcano plot representation of female hippocampal gene expression between the pair-fed control and alcohol groups.* In alcohol-exposed females, 25 hippocampal genes exhibited sex-specific alcohol-induced dysregulation, of which nine were upregulated and 16 were downregulated. Dotted lines denote selection criteria for significance ($p < 0.05$, and log2 (fold change) $\geq 2$) and separate differentially expressed genes and similarly expressed genes.

Among female offspring, Bioinformatic INGENUITY® Pathway Analysis (IPA®; Figure 17) identified dysregulation of 24 global biological pathways involving differential expression of hippocampal genes following chronic binge gestational alcohol exposure. IPA® determined the top canonical pathways dysregulated in ALC female hippocampi were proline biosynthesis I ($p = 0.0056$), regulation of Actin-based motility by Rho ($p = 0.0058$), PAK signaling ($p = 0.0079$), RhoA signaling ($p = 0.011$), and citrulline biosynthesis ($p = 0.012$).

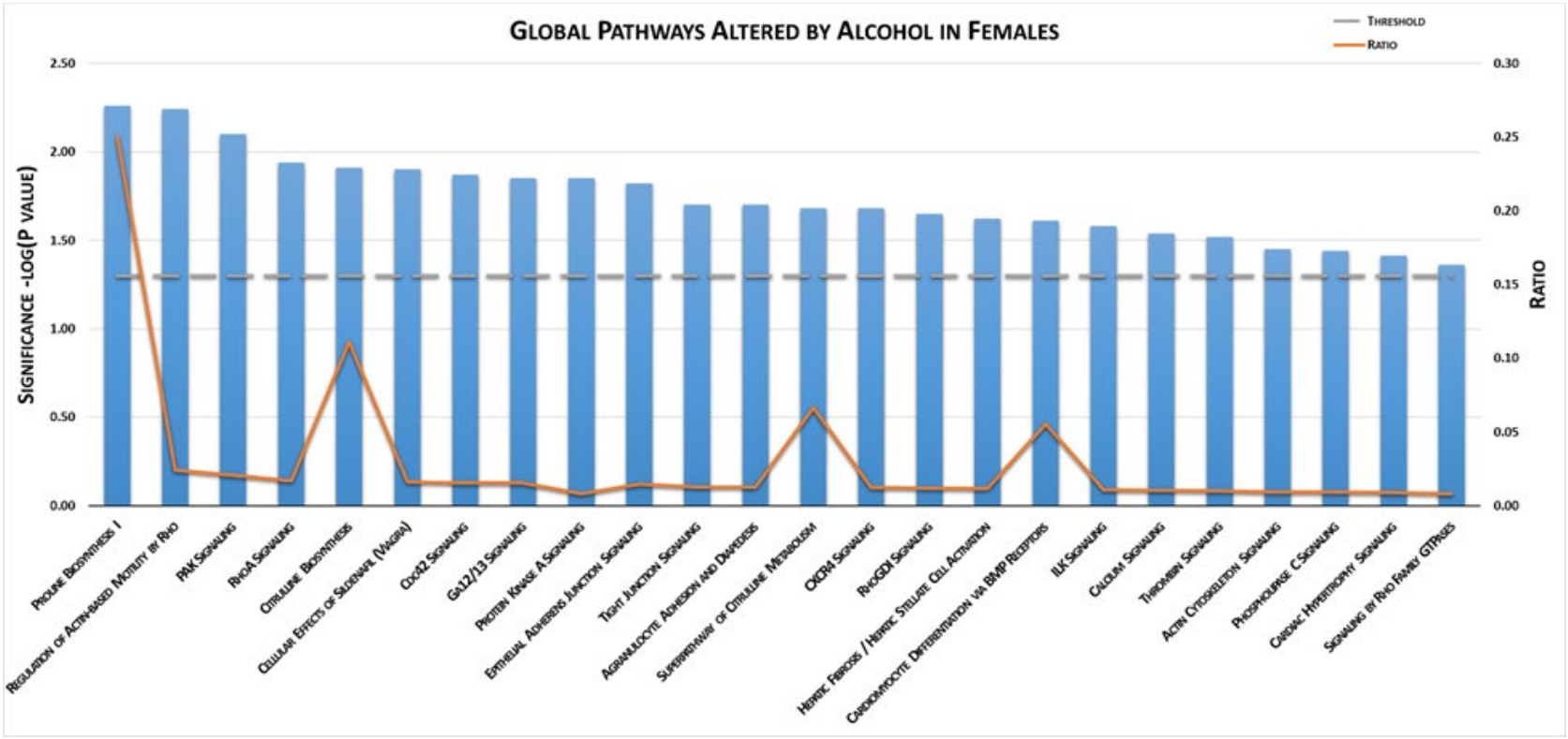


**Figure 17:** *INGENUITY® Pathway Analysis of female hippocampal differentially expressed genes.* In alcohol-exposed females, 24 global pathways were altered compared to the pair-fed control group ($p < 0.05$). Ratio represents the number of molecules affected to total number of molecules in each pathway.

Of the 28 male hippocampal genes exhibiting major changes described above, 12 genes showed upregulation and 16 exhibited downregulation (Figure 18). Seven of these dysregulated genes have a known gene-chemical interaction with choline: Aldehyde dehydrogenase 1 Family Member A3 (Aldh1a3, ↓), glutathione S-transferase, mu 5 (Gstm5 ↑), programmed cell death 5 (Pdcd5, ↑), RUN and FYVE domain containing 2 (Rufy2, ↓), saccharopine dehydrogenase (putative) (Sccpdh, ↑), sperm-associated antigen 4 (Spag4, ↑), SCO-spondin (Sspo, ↑), zinc finger protein 14 (Zfp14, ↓). Gstm5 (↑) and mitogen-activated protein kinase kinase 3 (Map2k3, ↓) have known gene-chemical interactions with ethanol. Other genes of interest include Sspo, involved in cell differentiation and nervous system development; reactive oxygen species modulator 1 (Romo1, ↑), involved in the response to reactive oxygen species, and mitochondrial fission factor (Mff, ↓), disease annotations which include developmental disabilities and mitochondrial encephalomyopathy.

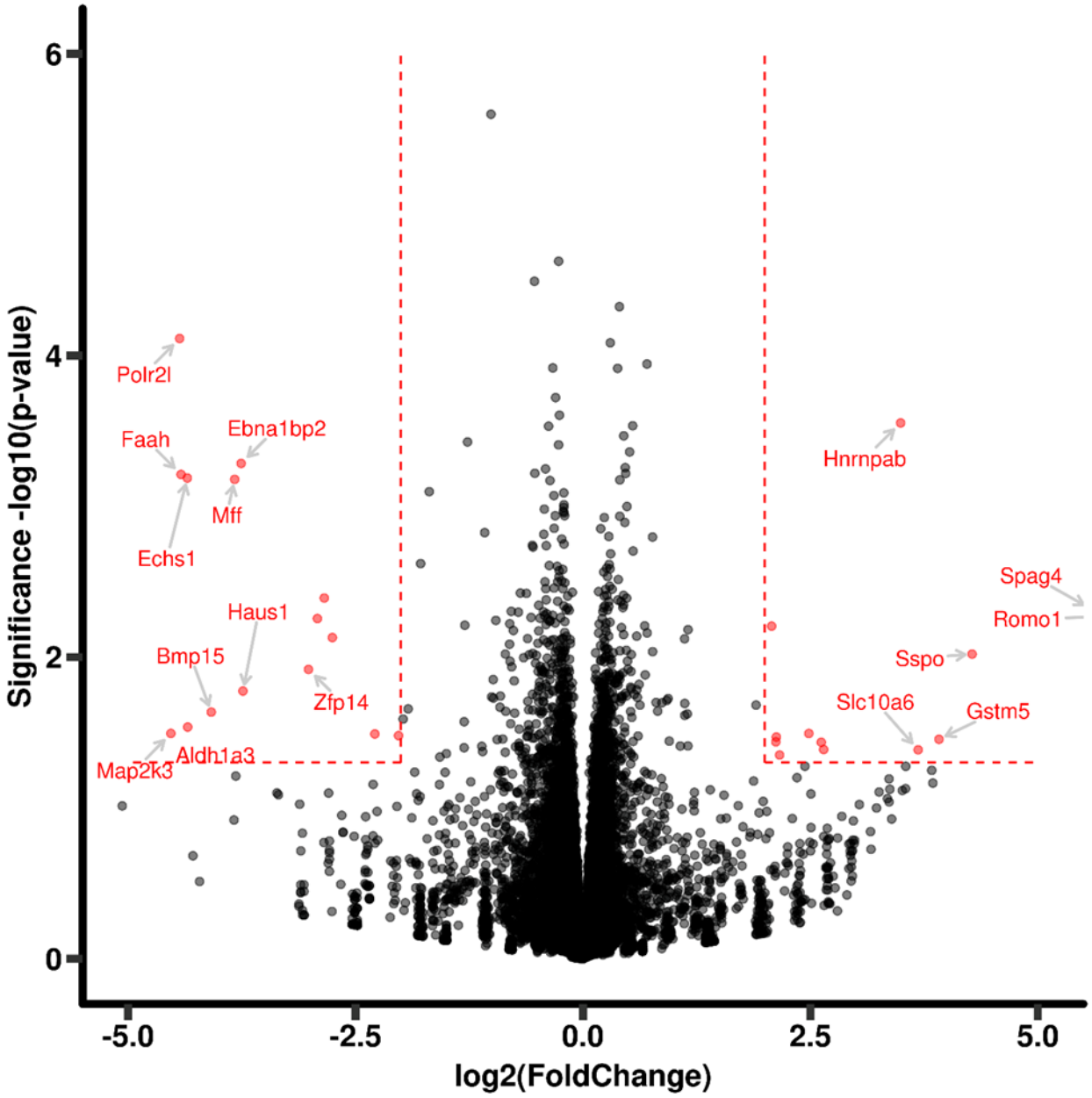


**Figure 18:** *Volcano plot representation of male hippocampal gene expression between the pair-fed control and alcohol groups.* In alcohol-exposed males, 28 hippocampal genes exhibited sex-specific alcohol-induced dysregulation, of which 12 genes were upregulated and 16 genes were downregulated. Dotted lines denote selection criteria for significance ($p < 0.05$, and log2 (fold change) $\geq 2$) and separate differentially expressed genes and similarly expressed genes.

Among male offspring, IPA® (Figure 19) identified dysregulation of 32 global biological pathways involving differential expression of hippocampal genes following chronic binge gestational alcohol exposure. IPA® determined the top canonical pathways dysregulated in ALC male hippocampi were xenobiotic metabolism signaling ($p = 0.0003$), anandamide degradation ($p = 0.0012$), alanine biosynthesis III ($p = 0.0012$), CD27 signaling in lymphocytes ($p = 0.0019$), and molybdenum cofactor biosynthesis ($p = 0.0049$).

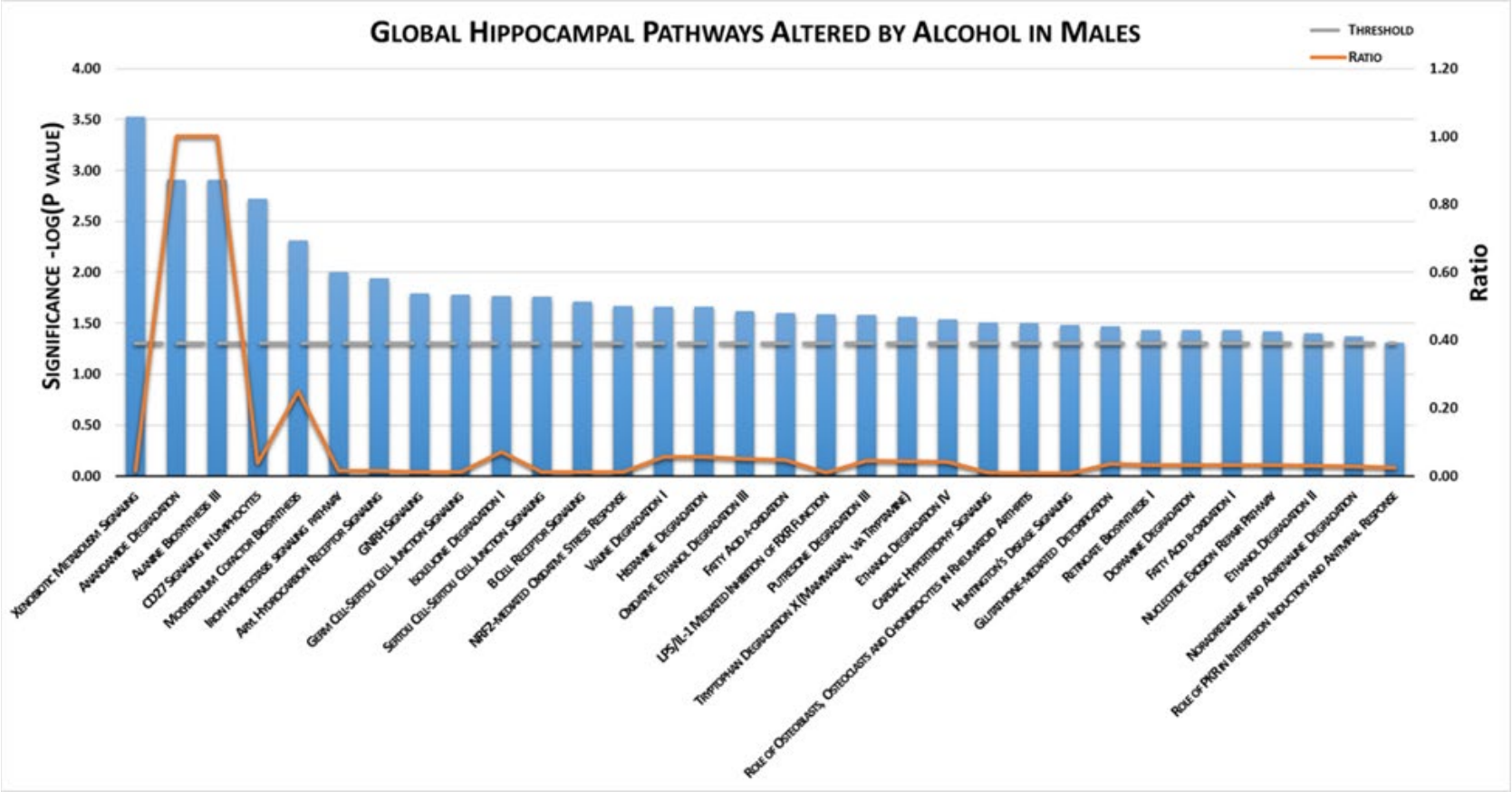


**Figure 19:** *INGENUITY® Pathway Analysis of male hippocampal differentially expressed genes.* In alcohol-exposed males, 32 global pathways were altered compared to the pair-fed control group ($p < 0.05$). Ratio represents the number of molecules affected to total number of molecules in each pathway.

## 5.4. Discussion

To our knowledge, this is the first investigation of the fetal hippocampal transcriptome utilizing next-gen high-throughput RNA-seq following chronic binge gestational alcohol exposure. Three salient findings can be gleaned from this study: (a) a chronic binge paradigm of gestational alcohol exposure dysregulates hippocampal gene expression, (b) this gene dysregulation manifests differently between male and female hippocampi, and (c) gene disruption following our exposure paradigm implicates key global pathways essential for healthy fetal development. Collectively, high-throughput RNA deep-sequencing identified 76 hippocampal genes with a significant expression difference between the PF and alcohol-treated groups, and within this group, a subset of 49 of these genes exhibited sex-dependent dysregulation.

Among female hippocampi, IPA® determined alcohol dysregulated 24 global canonical pathways following our chronic binge exposure, and includes the following pathways of interest: proline biosynthesis I, citrulline biosynthesis, and the superpathway of citrulline metabolism. Aldh18a1 encodes for the catalytic enzyme delta-1-pyrroline-5-carboxylate synthetase (P5CS), which is critical for de novo proline synthesis. Emerging data implicate proline's critical role as a neuroprotectant[49,50] through opposition to intracellular accumulation of reactive oxygen species[51,52], which has been extensively documented as a response to alcohol exposure in the developing brain. Taken in conjunction with proline's established roles as an antagonist to abiotic stressors[53–55] and an apoptotic regulator[56,57], alcohol-induced dysregulation of proline biosynthesis I may contribute to alcohol's pathogenesis in the developing hippocampus. Interestingly, Aldh18a1 is also critically implicated in citrulline biosynthesis and the super pathway of citrulline metabolism. Citrulline biosynthesis occurs downstream from the amino

acid precursors glutamate, proline, and arginine, and as arginine is converted to citrulline, nitric oxide (NO) is produced. Interestingly, NO is essential for healthy physiological nervous system regulation and has been shown to have critical roles in synaptic plasticity, learning, and memory[58,59]. It is possible that alcohol-induced dysregulation of citrulline-related biochemical pathways observed in the female hippocampus is reflective of dysregulation of nitric oxide synthase (NOS) activity in this region. Aldh18a1 downregulation among females may lead to accumulation of its substrate, glutamate, implicating a role for amino acid homeostasis in female hippocampal FASD pathogenesis. Although Aldh18a1 dysregulation has been linked with learning disabilities and neurodevelopmental deficits, hippocampal dysregulation in the context of FASD remains unknown.

Other genes of interest dysregulated by alcohol among female hippocampi include Mir410, Myl2, Phkg2, Pcsk1, and Slc6a13. Two downregulated genes, Myl2 and Phkg2, have gene-chemical interactions with ethanol, but to our knowledge have not been previously linked with FASD. Phkg2, a regulator of neural and hormonal regulation of glycogen breakdown, has been shown to be downregulated in whole-brain analysis following prenatal alcohol exposure, but has not been localized to the hippocampus in FASD[60]. Pcsk1, a highly expressed gene in the hippocampus that encodes for a serine protease responsible for processing neuropeptides and prohormones, has previously been identified as involved in brain development and is implicated in neuroendocrine signaling[48]. In Alzheimer's patients with severe neurodegeneration, the hippocampus is the most vulnerable region of Pcsk1 dysregulation[61]. Neu2, for which response to ethanol is a biological process, has shown dysregulation in human embryonic stem cells exposed to alcohol[62] and has been shown to be dysregulated in humans with alcohol dependence[63]. Slc6a13 (solute carrier family 6 member 13), which is involved in

neurotransmitter transport and binding, has also been linked by multiple reports with alcohol use disorders[64–66], but to our knowledge, its relationship to FASD has not been explored.

Among male hippocampi, IPA® determined alcohol dysregulated 31 global pathways following our chronic binge exposure. Pathways of interest include xenobiotic metabolism signaling, anandamide degradation, alanine biosynthesis III, and molybdenum cofactor biosynthesis. Xenobiotic metabolism signaling describes a cellular stress response to xenobiotic exposure and a concomitant metabolism response to detoxify drugs and other organic compounds[67]. Genes that were differentially expressed by alcohol and that are associated with this pathway include Aldh1a3, Gstm3, Map3k7, Map2k3. Interestingly, Gstm3 and Map2k3 have known gene-chemical interactions with ethanol. Gstm3 is a major detoxification enzyme shown to play a role in the breakdown of xenobiotics including a wide array of drugs and genetic variation is reported to influence susceptibility to toxins[68,69]. Recent microarray analysis reported dysregulation of glutathione pathways in the synaptoneurosome transcriptome of the mouse amygdala following a chronic alcohol exposure[70]. Anandamide is an endogenous neurotransmitter and Faah, a key gene within this pathway chiefly responsible for enzymatic breakdown of anandamide, was dysregulated by alcohol. Anandamide dysregulation is associated with hippocampal-based memory in rats and has been previously speculated to underlie FASD behavioral pathology[71,72]. Molybdenum cofactor biosynthesis and alanine biosynthesis III are directly related, and cysteine desulfurase (Nfs1) is implicated in each. Xanthine oxidoreductases are a class of molybdenum cofactor enzymes implicated in cellular responses to senescence and apoptosis[73], and the conversion of cysteine to alanine (alanine biosynthesis III) through sulfuration of xanthine oxidoreductase renders this class of enzymes catalytically active[74]. In humans, dysregulation of this process is associated with progressive

neurological damage[75]. Collectively, these pathways and their associated genes previously implicated in critical neurodevelopmental processes may play a role in FASD hippocampal pathogenesis observed in male offspring.

Other genes of interest dysregulated among males include: Sspo, involved in cell differentiation and nervous system development and has previously been identified as differentially expressed in autism spectrum disorders, bipolar disorder, and schizophrenia[76–78]; Romo1, involved in the response to reactive oxygen species and TNF-induced apoptosis[79–81]; and Mff, which is essential for embryonic development and synapse formation disease and annotations for which it include developmental disabilities and mitochondrial encephalomyopathy[82]. Seven dysregulated genes (Aldh1a3, Gstm5, Pdcd5, Rufy2, Sccpdh, Spag4, and Sspo) have a known gene-chemical interaction with choline. Prenatal alcohol-induced dysregulation of choline bioavailability is associated with impaired hippocampal development, learning, and memory[83], and we conjecture that these choline-interacting genes play an underlying role in this established alcohol-induced neuropathology. Though these genes have been previously implicated in alcohol-related neurological dysfunction, their roles in FASD hippocampal deficits remain to be explored.

Sex-based differences identified in the brain, and specifically in the hippocampus, have been shown to differentially affect susceptibility to disease, neurological function, and behaviors [84]. Collaborative reports investigating FASD models have implicated abundant alcohol-induced sex-specific hippocampal effects. Hippocampal neuroimmune response measured in offspring on PND 5 and 8 demonstrated a sex-dependent response to a developmental alcohol challenge[85]. Adolescent hippocampal functional assessment revealed N-methyl-D-aspartate long-term potentiation reduced by 40% in adolescent males prenatally exposed to alcohol compared to

adolescent females; interestingly, females exhibited increased hippocampal glutamine synthetase expression[86]. Prenatal alcohol exposure has also been shown to have sex-specific hippocampal effects lasting into adulthood, as Uban and colleagues demonstrated that PND 60 females exhibit a reduced proportion of newly produced neurons and glia in the dentate gyrus compared with males[87]. These studies indicate that alcohol has the potential to affect this brain region differentially based on sex, however, a knowledge gap persists regarding differences in hippocampal gene expression profiles between male and female rats alone and even more in the context of FASD[31,88,89]. An understanding of these differential outcomes at the transcriptome level is fundamental for developing novel therapeutic strategies that account for these sex-based differences for maximum effectiveness.

### 5.4.1. Perspectives and Significance

Thus far, the effects of chronic binge gestational alcohol exposure on hippocampal transcriptome-wide gene expression have remained largely limited to microarray analyses. The majority of hippocampal microarray analyses in animal models of FASD has been performed on: (a) adolescent or adult animals exposed to alcohol during development[27,28,30], or (b) on animals whose exposure paradigm did not mimic a chronic binge exposure paradigm throughout pregnancy[29]. To our knowledge, no microarray has analyzed rat hippocampal gene expression using a chronic binge model of gestational alcohol exposure. By utilizing next-gen high-throughput RNA-seq, our goal was to elucidate the novel molecular targets underlying FASD hippocampal deficits to better understand FASD pathogenesis. In summary, our results indicate that a chronic binge paradigm of gestational alcohol exposure differentially alters the hippocampal gene expression, and that this alcohol-induced gene expression exhibits sex-

specific variation in the developing hippocampus, as do their associated global canonical pathways. Detection of subtle gene expression changes within specific brain regions, such as the hippocampus, through advances in next-generation sequencing may yield critical new understanding of vulnerable genes and genetic networks underlying FASD neuropathogenesis. Insights acquired from this advanced genomic technology offer novel findings essential for pinpointing targets of developmental alcohol exposure necessary for the development of urgently needed, targeted therapeutic intervention strategies.

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# 6. DISCUSSION

Maternal uterine vasculature is an under-explored area of FASD investigation. In Chapters 2 & 3, we aimed to bridge this knowledge gap closer by exploring how chronic binge alcohol exposure influences the uterine artery's ability to adapt to pregnancy. We focused on the maternal primary uterine artery because of its vital role in sustaining fetal growth and development via oxygen and nutrient delivery, particularly since impaired fetal growth and development are FASD hallmarks. The overall finding from analyzing this profoundly influential blood vessel is that alcohol affects not only fetal health but maternal health, specifically uterine artery functional adaptations, and alcohol-mediated changes in the mother may play an influential role in fetal health outcomes accompanying FASD.

## 6.1. Alcohol-Mediated Vascular Dysfunction is Linked with Growth Restriction

The first major finding from the preceding vascular studies was that these data uniquely link alcohol-mediated uterine artery dysfunction with fetal growth restriction (FGR). Following our chronic binge prenatal alcohol exposure (PAE) paradigm with clinically relevant BACs, mean fetal body weight of alcohol-exposed animals decreased by nearly 16% compared with controls. FGR is a hallmark feature of FAS, the most severe form of FASD, and has been described in the literature since the earliest fetal alcohol studies[1,2]. Irrespective of alcohol, FGR is widely recognized in clinical practice as an reliable indicator of compromised fetal and neonatal health, as it correlates with increased morbidity and mortality and other serious health complications at birth (e.g., low Apgar scores, hypoxia, hypoglycemia, hypothermia, meconium aspiration, and higher infection risk, etc.)[3,4]. Low birthweight has also been linked with long term

health complications that present in and may persist throughout adulthood (e.g., hypertension and diabetes)[5], so much so that a working hypothesis suggests it may be a root source for chronic diseases that present in adulthood. Other FASD hallmarks, such as behavioral and neurological deficits (e.g., cognitive development, speech pathology), have been closely associated with FGR alone[6–10]. Based on this and the consequential nature of FGR, it is likely that it may influence the presentation and severity of other FASD deficits.

In other at-risk pregnancies not in the context of alcohol (e.g., those with preeclampsia, diabetes mellitus, hypertension, etc.), uterine artery dysfunction has been shown to accompany fetal growth restriction (FGR)[11–13]. In fact, the leading cause of non-FASD FGR is "reduced uteroplacental perfusion associated with maternal vascular disease" and may be the underlying mediator for up to 25–30% of FGR patients[3]. Currently the mechanism(s) for alcohol-mediated FGR remains unknown. We posit that FGR observed in this study and in FASD patients, at least in part, derives from alcohol-mediated uterine artery dysfunction, which may diminish the uterine artery's capacity to meet fetal metabolic demand.

### 6.2. Alcohol Impairs the Uterine Artery Myogenic Response

The second major finding of this work was that alcohol alters the uterine artery's ability to adjust to changes in transmural pressure, or its myogenic response. The main task of the uterine artery during pregnancy is to maintain perfusion to the uteroplacental compartment, and the myogenic mechanism is an essential vascular adaptation for ensuring this[14,15]. This autoregulatory mechanism present in the walls of afferent vessels is not mediated by innervation but instead is locally controlled by the degree of mechanical stretch applied to vascular smooth

muscle cells generated by changes in transmural pressure (Bayliss effect)[16] and by circulating and local metabolite levels[17,18]. Arterial wall smooth muscle maintains optimal vessel diameter by increasing its tension via contraction in response to transmural pressure elevation or by decreasing its tension via relaxation in response to pressure decline[19]. A proposed mechanism for this suggests an increase in mechanical stretch leads to ion channels in the cell membrane physically opening, which in turn stimulates voltage-gated calcium ($Ca^{2+}$) channels, raising cytosolic $Ca^{2+}$, depolarizing the smooth muscle cell, and initiating contraction, whereas less stretch leads to the opposite response and results in smooth muscle relaxation[20–23]. The myogenic mechanism allows arteries and arterioles to adjust to physiological stressors in real time, thus tightly regulating resistance, and in turn adjusts blood flow in these afferent vessels according to the metabolic needs of the organs they supply[24–26]. What exactly occurs to the uterine artery myogenic response during pregnancy is not definitive[27,28], but studies suggest it generally diminishes during pregnancy under normal conditions[20,24,29–32] and increases under pathological conditions such as undernutrition, impaired perfusion, and chronic hypoxia[33–36]. What is definitive is that the myogenic mechanism is essential for regulating maternal hemodynamics that support healthy fetal development. Very limited knowledge exists on how alcohol affects the uterine artery myogenic response in pregnancy.

Using a pressure myograph approach controlling for some *in vivo* parameters (e.g., pH, temperature, osmolarity), we cannulated near term (GD 20) uterine arteries from control and alcohol-exposed groups and measured whole vessel and vessel lumen diameters as transmural pressure was increased in 20 mm Hg increments. Arterial diameters from the control group initially decreased at lower increasing pressure increments (40-60 mmHg) as basal tone developed, but at physiological pressures (80-120 mm Hg), metrics were essentially unchanged.

This confirmed that the myogenic response was present, even in excised vessels, otherwise increasing pressure would have elicited distension. It could be reasoned that if flow were present in these vessel segments under normal physiological conditions, flow would be maintained, since diameter and consequent resistance were unaffected at *in vivo*-like pressures.

In arteries from alcohol-exposed pregnant rats, our data indicated a myogenic response was similarly present based on their comparable reactivity to increasing pressure. However, alcohol diminished uterine artery vessel ($P = 0.0025$) and lumen ($P = 0.0020$) diameters, suggesting greater transmural pressure-dependent constriction, and this effect was most evident at physiological pressures (80-120 mm Hg). If we apply Poiseuille's law to hemodynamic context, vascular resistance is inversely proportional to vessel lumen radius to the 4$^{th}$ power[37]. Thus, alcohol-mediated reduction of uterine artery diameter would profoundly reduce blood flow into the fetal compartment, the uterine artery's chief purpose during pregnancy.

It is possible that the uterine artery normally transitions to a reduced vascular tone via diminished myogenic response during pregnancy and that what occurred in the alcohol group is failure of that reduction to occur. Although studies lack concordance as to what happens to uterine artery myogenic behavior in pregnancy, an established way that maternal physiology adapts to pregnancy is by increasing cardiac output to boost cumulative uteroplacental perfusion[38]. This is achieved by decreasing systemic vascular resistance and blood pressure and increasing blood volume and heart rate[15,24,39]. This drop in vascular resistance is not entirely generalized but is strategic so that up to 20% of maternal cardiac output is rerouted to the fetal compartment[14]. If alcohol impairs this decline in vascular resistance, as shown here in the uterine artery's response to pressure but does not negate a naturally occurring fall in blood pressure, blood flow to the fetal compartment could be dramatically reduced.

It is also possible that alcohol increases the sensitivity of the myogenic response such that smooth muscle in alcohol-exposed uterine arteries responds to increased pressure by generating added tension, therefore eliciting a greater contractile response than that observed without alcohol exposure. One way this could be achieved is if alcohol had an effect on activating enzymes responsible for regulating $Ca^{2+}$ sensitivity within the cell. In non-uterine resistance arteries (e.g., cerebral, mesenteric), activation of protein kinase C (PKC) and rho-associated protein kinase (ROCK) have been shown to augment myogenic contraction by increasing sensitivity to intracellular $Ca^{2+}$[40–43], possibly by myosin light chain phosphatase (MLCP) inhibition[44,45]. If $Ca^{2+}$ sensitivity is increased, vascular smooth muscle can produce greater myogenic contraction even with a reduction in intracellular $Ca^{2+}$[46]. This could be a theoretical alternative for interpreting the uterine artery behavior in the alcohol group.

### 6.3. Alcohol Influences Vascular Remodeling

The third major finding reported here was that alcohol modified the type of vessel remodeling the uterine artery underwent. During pregnancy, the uterine vasculature undergoes dynamic structural transformation achieved by hypertrophy (i.e., smooth muscle cell enlargement) and hyperplasia (i.e., endothelial cell proliferation)[47]. Structural remodeling is a key vascular adaptation for the uterus accommodating an increase in uterine blood flow up to 50-fold at parturition[38,48]. It is also a physiological phenomenon unique to this vascular network, as the structure of arterial beds supplying other organs is, at least from a macro perspective, relatively static throughout life and if major structural change occurs, it is often pathological (e.g., atherosclerosis)[14]. Generally, the structural remodeling that occurs in the uterine artery during pregnancy is termed as outward hypertrophic remodeling, characterized by an increased

lumen diameter and a decreased media (vessel wall thickness) to lumen diameter (media:lumen) ratio[14,15]. This means that lumen cross sectional area increases and the arterial wall thickness is essentially unchanged, resulting in a larger volumetric space for conducting blood flow and, consequently, reduced vascular resistance. Under normal physiological conditions, blood vessel length generally remains fixed[14,39,49]. However, uterine vessels also lengthen substantially during pregnancy, which increases resistance and opposes blood flow[14,15,39,47]. Per application of Poiseuille's law, the relationship of vessel length with resistance is directly proportional and linear, compared to the relationship of lumen radius (and therefore diameter) to resistance, which is inversely proportional and quadratic[15]. This means that with respect to uterine artery remodeling during pregnancy, lumen enlargement has a much more influential effect on resistance than vessel lengthening, resulting in a net reduction of uterine vascular resistance and in blood flow augmentation to the maternal-fetal interface.

Our data demonstrated that alcohol transforms uterine artery remodeling from outward hypertrophic remodeling to inward hypotrophic remodeling, which is characterized by decreased lumen diameter and an increased media:lumen ratio. These parameters imply that compared to controls, uterine arteries from animals chronically exposed to alcohol during pregnancy had less capacity for blood flow conduction and that their vessel walls may have thickened. Studies show that this type of remodeling increases arterial resistance[50,51], has been observed in patients with hypertension[52–57] and in arteries with chronically reduced blood flow[58], and has been linked with target organ damage[59,60]. Inward hyportrophic remodeling may be an auxiliary factor that increases uterine artery resistance in addition to alcohol-mediated myogenic changes. Studies outside the context of alcohol describe the inability of arteries to correctly remodel or maintain their essential structure as a type of vascular insufficiency and that is functionally analogous to

other organ insufficiencies (e.g., cardiac, renal)[54,60]. Our data indicate that alcohol may be a mediator for uterine artery vascular insufficiency in pregnancy based on how it modifies vascular remodeling.

Additional studies are needed to validate and holistically assess the type of structural changes occurring in alcohol-exposed uterine arteries that our data suggest. For example, the term hypertrophy implies cell enlargement, but it is possible the observed wall thickening is instead occurring because of greater smooth muscle contraction from an elevated myogenic response and is not a truly hypertrophic event. Alternatively, the uterine artery wall may only appear to be thickened, but in actuality, the tissue comprising the vessel wall has the same amount of mass but is accommodating a much smaller lumen, so that even if the wall is measurably thicker, the amount of tissue surrounding the arterial lumen remains the same.

Whether alcohol is a direct or indirect mediator of vascular remodeling is unknown. It is possible alcohol directly influences uterine artery remodeling by altering either local molecular signaling processes in the vessel wall that affect vascular growth [e.g., vascular endothelial growth factor (VEGF)], elasticity of the connective tissue in the laminae and extracellular matrix, or smooth muscle activity. However, many other factors impact uterine artery structural remodeling and subsequent function during pregnancy, including upstream and downstream structural changes (e.g., vascular branching, angiogenesis), physiological elements (e.g., cardiac output, blood pressure, hormone signaling), hemodynamic parameters (e.g., characteristic impedance, shear stress, blood viscosity), and even fetal processes (e.g., placental efficiency, fetal metabolism)[37,47,54]. Despite their heterogeneity, these factors (and others) are capable of affecting vascular remodeling in some way. Alcohol could influence any of these factors to varying degrees, possibly even in addition to having direct local effects on remodeling.

Histological investigation would be a logical next step for identifying what changes occur in each of the wall components before scouting up and downstream etiologies. Failure to validate that the arterial wall has in fact thickened post alcohol exposure either histologically or via a filar micrometer during pressure myograph studies limits the scope of this study.

These combined factors suggest that PAE increases the predominant hemodynamic force (resistance) that the maternal uterine artery must overcome to maintain perfusion, which directly opposes its intended purpose of delivering oxygen and nutrients to the fetal compartment at an ever-increasing demand. It has been shown that alcohol may decrease uterine blood flow up to 40%[61], and these alcohol-mediated effects on uterine artery adaptations may be an underlying reason for this reduction. In theory, if the uterine artery is unable to compensate for the amplified resistance, fetal metabolic demands may not be met, resulting in adverse health outcomes associated with FASD.

Next, we took a closer look at how alcohol mediates uterine artery vascular dysfunction at the level of the endothelium and the implications this has on uterine artery basal tone, i.e., the intrinsic smooth muscle tone at set physiological conditions. While the myogenic response mechanism is thought to be primarily stimulated by mechanical stretch, studies show that under certain physiological conditions and in response to certain stimuli, the endothelium regulates vascular tone via production and activation of several substances known as endothelial-derived vasodilators and that these increase during pregnancy[24,29,31,62–64]. Functional alterations to the endothelium of the reproductive vasculature are a necessary adaptation for supporting a healthy pregnancy[24,27,49,65,66], and prior works suggest the endothelium is vulnerable to alcohol in non-reproductive vasculature[67–72]. We reasoned that alcohol damages the uterine artery endothelium

by disrupting endothelial-derived vasodilators and that this may be a contributing mechanism to our observed alterations in basal tone.

### 6.4. Alcohol Impairs Endothelial-Dependent Vasodilation

The next major finding from these vascular studies was that alcohol impairs agonist-mediated vasodilation that is endothelial-dependent. The endothelium was historically considered a simple cell layer, lining blood vessels, and acting as an inert, clot-resistant, barrier between blood and supplied tissues[73,74]. Only relatively recently has it received appropriate recognition as being a dynamic structure with complex actions that regulate a range of homeostatic processes[73,74]. Endothelial functions include:

1) transport of simple ions and complex molecules (e.g., glucose, amino acids, albumin),
2) synthesis and metabolism of signaling molecules that influence local and remote cellular processes (e.g., cytokines [such as chemokines, growth factors, interleukins, and interferons], pro- and anti-clotting factors, vasoactive factors, etc.),
3) clot formation (e.g., endothelial expression of tissue factor receptors activates factor X and initiates the clotting cascade),
4) immune/inflammatory responses (e.g., endothelial cells express adhesion molecules that regulate leukocyte migration into tissues, and
5) vascular processes (e.g., tone regulation, angiogenesis)[73,74].

Endothelial receptors may couple with neurotransmitters (e.g., Ach), hormones (e.g., estrogen, progesterone), metabolites, and other vasoactive factors to regulate vascular tone, which in turn directly influences key hemodynamic parameters such as systemic vascular

resistance and blood flow[73,74]. The endothelium is dynamic in that cells can vary in their receptor expression and responses to certain stimuli based on their location in the body[73,74]. In other words, blood vessels behave differently in different parts of the body in part due to local variation of protein expression among endothelial cell populations. This phenotypic heterogeneity ensures that nutrient and oxygen delivery is tightly regulated in real time and on demand[73]. This endothelial feature also accounts for how the uterine artery can undergo extensive transformation and behave so differently from the non-pregnant to pregnant state[24,64,75,76]. Based on this, it is reasonable to infer that the uterine artery may have a unique response to alcohol compared to arteries elsewhere in the body. Contingent on the uterine artery's unique adaptability and on the previous findings that alcohol alters uterine artery responses to pressure and remodeling, a closer look at how alcohol influences endothelial activity in this vessel was warranted.

To assess alcohol effects on uterine artery endothelium, excised uterine artery segments from control and alcohol-exposed animals were maintained in an arteriograph chamber as described in Chapter 2, except that after an equilibration period, intralumenal pressure was maintained at 90 mmHg (approximating mean arterial pressure in both rats and humans) and intrinsic tone was achieved. Vessels were then pre-constricted using thromboxane (Tbx), an eicosanoid and derivative of arachidonic acid. In the body, Tbx is synthesized in platelets, and it derives its name from the Greek words *thrombus* (blood clot) and *thrombosis* (curdling) for its role in doing just that – facilitating blood clot formation by activating and promoting aggregation of platelets[77]. However, Tbx is also a potent vasoconstrictor, which also supports the clot formation process[77,78]. Tbx binds to thromboxane prostanoid (TP) receptors on cell membranes

of vascular smooth muscle cells, which are a variety of G-protein coupled receptors, and elicits contraction primarily by one of two ways:

1) Tbx binding to a TP with a Gq subunit activates membrane-bound phospholipase C (PLC), triggering a second messenger signaling cascade that yields inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG), which stimulates $Ca^{2+}$ efflux from the smooth endoplasmic reticulum into the cytoplasm and PKC activation[77–79]. $Ca^{2+}$ elicits smooth muscle contraction by binding to the protein calmodulin, and this complex activates myosin light chain kinase (MLCK), which phosphorylates myosin light chain (MLC)[77–79]. PKC promotes smooth muscle contraction in several ways:
    a. CPI-17 phosphorylation inhibits MLCP and increases MLC phosphorylation, and thereby, increases $Ca^{2+}$ sensitivity of the contractile apparatus,
    b. potassium ($K^+$) channel inhibition leads to membrane depolarization and voltage-gated $Ca^{2+}$ channel opening/activation, and
    c. by initiating a protein kinase signaling cascade that phosphorylates caldesmon, a protein that binds actin and augments contraction[80].
2) Tbx binding to a TP with a G12/13 subunit ultimately phosphorylates ROCK, which activates MLCK and this enzyme in turn phosphorylates MLC[77–79], also increasing $Ca^{2+}$ sensitivity.

Preconstriction of artery segments is a common practice in pressure myography to generate a baseline diameter from which to assess diameter change from and to measure the full range of vasodilatory response.

Subsequently, increasing concentrations of the agonist acetylcholine (Ach) were added to the chambers housing the preconstricted, pressurized vessels under identical conditions from

alcohol-exposed and pair-fed treatment groups. Ach acts on the endothelium by binding to M3 muscarinic receptors to stimulate vasodilation by 3 possible signaling pathways:

1) *Nitric oxide (NO)*: Ach binding to a G-protein coupled receptor causes a conformational change followed by PLC activation, which cleaves phosphatidyl inositol biphosphate into IP3 and DAG and initiates a second messenger signaling cascade[73,81]. IP3 stimulates $Ca^{2+}$ release into the cytoplasm from the smooth endoplasmic reticulum, raising endothelial intracellular $Ca^{2+}$ levels[73,81]. $Ca^{2+}$ binds to the cytoplasmic protein calmodulin, eliciting a conformational change that enables binding to inactive endothelial nitric oxide synthase (eNOS)[73,81]. It is theorized that in its inactive state, eNOS is bound to the protein calveolin-1 in cell membrane pockets called calveolae[81]. The binding of the $Ca^{2+}$-calmodulin complex with inactive eNOS triggers detachment of eNOS from its calveolin-bound position, activating the enzyme[73,81]. In contrast to vascular smooth muscle, elevated $Ca^{2+}$ in the endothelium leads to vasodilation via the action of NO produced by eNOS in the endothelium[73]. Activated eNOS converts L-arginine to NO and L-citrulline, and NO is then transferred into neighboring smooth muscle cells via simple diffusion, where it acts locally to stimulate soluble guanylyl cyclase (sGC) to convert guanylyl triphosphate (GTP) into cyclic guanylyl monophosphate (cGMP)[73,81]. cGMP acts as a second messenger on protein kinase G, which in turn inactivates MLCK by phosphorylating it, yielding MLCK phosphate (MLCK-PO4), thus preventing vascular smooth muscle contraction and resulting in vasodilation[73,81]. cGMP also prevents $Ca^{2+}$ release from the smooth muscle sarcoplasmic reticulum and stimulates $Ca^{2+}$ uptake by this organelle as an additional vasodilatory action[73].

2) *Prostacyclin (PGI2)*: Upon Ach binding to the endothelial cell membrane, arachidonic acid is converted to prostaglandin H2 (PGH2) by cyclooxygenases (COX-1, COX-2), and PGI2 is synthesized from PGH2 by the enzyme prostacyclin synthase[73]. PGI2 acts in a paracrine manner on smooth muscle cells by binding to a PGI2 G-protein coupled receptor, initiating a second messenger cascade that increases cytosolic cyclic adenosine monophosphate (cAMP). cAMP activates PKA, which phosphorylates MLCK into its inactive form, MLCK-PO4, thereby inhibiting smooth muscle contraction and resulting in vasodilation akin to NO[73]. PGI2 can also hyperpolarize vascular smooth muscle cells by influencing the activity of various potassium channels[82].
3) *Endothelial-derived hyperpolarizing factor (EDHF)*: Ach also acts as an agonist for EDHF, an unknown vasodilatory substance(s) or signal released by the endothelium[82–86]. EDHF mediates vasodilation by hyperpolarizing neighboring smooth muscle cells by making resting membrane potential more negative[73,82–86]. EDHF was proposed after smooth muscle hyperpolarization still occurred in vessels with an intact endothelium and NO and PGI2 inhibition[83]. The mechanism for how EDHF induces vasodilation is unknown, but several theories have been proposed:
    a. Ach-mediated $Ca^{2+}$ increases in endothelial cells leads to $K^+$ efflux into the extracellular space, stimulating $K^+$ release from neighboring cells, resulting in their hyperpolarization. A more negative cell membrane potential also increases $Ca^{2+}$ reuptake from the cytoplasm in smooth muscle cells[82–84,86].
    b. Arachidonic acid metabolism in the endothelium by cytochrome P450 epoxygenase enzymes produces byproducts known as epoxyeicosatrienoic acids

(EETs) that mediate $K^+$ efflux in smooth muscle cells resulting in hyperpolarization[87].

c. Gap junctions between permit Ach-mediated hyperpolarization in the endothelium to be conducted to smooth muscle cells, and gap junctions connecting smooth muscle cells permit this hyperpolarization to spread throughout the tunica media[85].

d. Endothelial C-type natriuretic peptide (CNP) may hyperpolarize smooth muscle by G protein-gated KIR-channels (GIRK) and $Na^+$–$K^+$-ATPase activation[84].

Uterine arteries that underwent chronic binge alcohol exposure showed a 30% decrease in their ability to vasodilate compared with pair-fed controls. This response to Ach implies alcohol specifically disrupts one or more of the three vasodilatory pathways originating in the endothelium for which Ach is an agonist (i.e., NO, PGI2, EDHF).

The next logical step was to test whether the vasodilatory deficit was in fact endothelial dependent or independent. Theoretically, alcohol could directly alter the ability of vascular smooth muscle to dilate by altering its ability to receive or respond to vasodilatory stimuli or it could alter its sensitivity to these stimuli. To distinguish whether chronic binge alcohol resulted in endothelial or smooth muscle dysfunction, a separate experiment with identical treatment groups was performed where the endothelium was removed from cannulated uterine artery segments that were then similarly pressurized (90 mm Hg) and exposed to increasing concentrations of sodium nitroprusside (SNP).

SNP is a molecular complex of iron, cyanide, and nitrosonium ($NO^+$), and is administered IV to clinically treat hypertensive crisis and acute congestive heart failure[88,89]. It dissolves easily in circulation, and the ferrous component combines readily with sulfhydryl groups of

oxyhemoglobin in red blood cells to form cyanmethemoglobin, generating cyanide and NO as byproducts[88–90]. The cyanide is metabolized in the liver by the enzyme rhodanese into thiocyanate, which is then excreted by the kidney[88,90,91]. The NO acts immediately on vascular smooth muscle as a potent vasodilator, reducing systemic vascular resistance, cardiac preload and afterload, and mean arterial pressure[88,90]. In this study, there was no difference in the vasodilatory response to SNP between treatment groups, which conveyed that the vasodilatory deficit observed in the alcohol group was endothelial-dependent, justifying further investigation of the effects of alcohol on this specific tissue.

It is important to note the alignment of this finding with previous work linking impaired endothelial function heavy alcohol exposure. Historically, alcohol has a complex relationship with the entirety of the cardiovascular system, including the endothelium, as light to moderate alcohol consumption has been shown to have protective effects, such as reduced risk for ischemic stroke and coronary artery disease[67]. Other cardio-protective effects of drinking in moderation include significant increases in high-density lipoprotein cholesterol levels and decreasing platelet adhesiveness and aggregation, as well as other molecular factors that promote clot formation[92,93]. However, in parallel with PAE which demonstrates dose-dependent effects on phenotypic severity[94–96], vascular function exhibits profound vulnerability to both chronic and elevated levels of alcohol exposure. Excessive drinking has an established history with increasing risk cardiovascular risks such as hypertension, elevated triglycerides, and insulin resistance[97,98]. Binge drinking increases cardiac arrhythmias, including cardiac arrest[99,100]. With respect to the endothelium, a similar paradox has been observed. High alcohol exposure binge drinking can lead to endothelial injury and toxicity[67,72,101]. The current findings in this study support previous work determining higher alcohol doses can impair Ach response and

endothelial-dependent vasodilation in various vascular beds across species outside the context of pregnancy: mesenteric arteries in rats[102], cerebral resistance arterioles in rabbits[103,104], and brachial arteries in humans[105]. In conjunction with the clinical observation that FGR occurs most frequently in patients exposed to higher alcohol levels *in utero*[106,107], it is hypothetically possible that uterine artery endothelial dysfunction in response to chronic, binge-alcohol exposure may be a candidate mechanism underlying this phenotype.

### 6.5. Alcohol Disrupts NO Signaling

The next major finding of this work was that alcohol specifically impaired NO-mediated endothelium-dependent vasodilation in the uterine artery during pregnancy. The main reason why we focused this study on the role of the NO pathway in alcohol-mediated vasodilatory dysfunction over the other endothelial-derived vasodilatory pathways (PGI2/EDHF) was based on substantial investigative overlap previously established between NO with pregnancy and with vascular remodeling, and with alcohol, individually. However, very little is known about how alcohol influences this pathway in maternal uterine artery and what this means for fetal outcomes.

NO is a powerhouse with respect to its versatility in supporting homeostatic processes[108,109]. In pregnancy, NO plays prolific roles in mediating vascular adaptations that in turn sustain healthy fetal growth and development[108]. Since the vascular bed supplying the feto-placental compartment is not innervated by the autonomic system, local NO cell signaling is a key determinant in blood supply to this structure[108]. In early pregnancy, NO facilitates implantation, cytotrophoblast invasion, and angiogenesis[109]. Human and animal models suggest

NO activity augments the hemodynamic changes that coincide with pregnancy by lowering systemic vascular resistance[75,110,111] and moderating decreases in blood pressure[112]. Across species, the essential nature of NO in pregnancy is evidenced by upregulation of eNOS and iNOS expression in various tissues and is corroborated by elevated cGMP in plasma and urine samples, as well as elevated NO metabolites in circulation[113,114]. In the sheep model, NO release has been shown to correlate directly with uterine artery endothelial-mediated vasodilation in late pregnancy[115].

Another reason why NO pathway exploration is particularly relevant is because NO is a key moderator for vascular remodeling, specifically circumferential enlargement, an essential hemodynamic adaptation to pregnancy[116]. We know that outside of pregnancy, eNOS and NO deficiencies of have been linked with reduced capacity for vascular remodeling in various organ systems and with pathological changes to vessel structure that have been associated with such diseases as hypertension and atherosclerosis[54,55,116,117]. In pregnancy, NO is a master regulator for uterine and placental vascular remodeling, and this remodeling is highly adaptive, dynamic, and ever-increasing based on fetal growth and metabolic demand[15,47,118]. Across species and in both gravid and non-gravid states, vascular diseases linked with NO dysregulation often promote vessel reconstruction that is characterized by decreased lumen diameter and increased wall thickness[116,119–122]. This aligns with the type of remodeling (inward hyportrophic) described in the prior experiment and supports investigation of whether alcohol-induced NO disruption could be a mediator underlying these structural changes to uterine arterial wall structure.

Alcohol is a well-known influencer of NO signaling throughout the body's vascular landscape. In alignment with the inherently complex nature of alcohol's actions described above on the endothelium, its influence specifically on NO signaling in the endothelium is duplicitous:

generally low concentrations have shown favorable effects on endothelial function, whereas high doses have proven detrimental[101]. Other factors, such as exposure duration and vascular bed location also play a role in alcohol's influence on NO signaling as it pertains to endothelial functional endpoints[101,123]. This complexity as well as all three endothelial vasodilatory pathways having shown vulnerability to alcohol pose challenges to the next step in our study. We chose to investigate NO over the PGI2 and EDHF pathways for several reasons. Studies have identified NO susceptibility to alcohol in reproductive-adjacent vasculature, including spiral arteries of the uterus and in the placenta[101,124,125]. Both maternal and fetal systemic arteries have shown NO dysfunction in response to alcohol exposure during pregnancy[101,123,126,127]. An additional reason is that during pregnancy, the contribution of the individual components of the endothelial-derived vasodilatory pathways has been shown to shift, suggesting that NO signaling plays a more prominent role in modulating vasodilation[126]. It has been suggested that this shift may amplify the effects of alcohol on vascular tone[126].

Identifying whether alcohol disrupts the NO-mediated endothelial-derived vasodilation is acutely relevant based on the collective importance of both NO and the uterine artery for sustaining pregnancy and on the potential for harm that alcohol-mediated NO disruption could have on fetal outcomes. To determine this, uterine arteries were cannulated as described in the previous study, and then were incubated with inhibitors of both the PGI2 (indomethacin) and EDHF (apamin, TRAM 34) pathways, leaving the NO pathway intact:

1) Indomethacin blocks PGI2 production by inhibiting COX-1 and -2, the upstream enzymes responsible for PGI2 synthesis.
2) Apamin is a neurotoxin naturally occurring in bee venom that selectively binds and blocks small conductance $Ca^{2+}$-activated $K^{+}$ channels.

3) TRAM-34 (1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole) is an analog of clotrimazole that selectively inhibits intermediate -conductance $Ca^{2+}$-activated $K^{+}$ channels.

After incubation in a circulating buffer with these added inhibitors, arterial segments were similarly preconstricted with Tbx and their vasodilatory response to Ach was reassessed. A diminished relaxation response in both treatment groups was foreseeable, as two thirds of the endothelial-derived vasodilatory pathways were rendered non-functional by selective inhibition. Indeed, vascular relaxation was attenuated in both alcohol and control groups compared with their respective pre-inhibition response traces. However, the magnitude of post-inhibition relaxation reduction was nearly equal (~47%) between treatment groups, indicating a lack of and/or an equal alcohol effect on the collective PGI2 and EDHF pathways. In other words, since PGI2/EDHF inhibition failed to show an alcohol effect, the alcohol effect shown prior in the Ach response study sans inhibition likely implicates the remaining NO pathway. This implication was further supported by the lack of alcohol effect in the SNP response described earlier. Overall, these findings suggest that alcohol impairs endothelial-mediated vasodilation by disrupting the NO pathway.

To support this indication that uterine artery endothelial NO signaling is acutely vulnerable to alcohol exposure, levels of endothelial nitric oxide synthase (eNOS), the enzyme responsible for endothelial synthesis, as well as of eNOS phosphorylated at the site of the excitatory serine residue 1177 ($^{P\text{-}Ser1177}$eNOS), were assessed in uterine arteries from alcohol-exposed and control treatment groups. Presumably because NO is a profoundly potent vasodilator, the enzyme responsible for its production is regulated at transcriptional, post-transcriptional, and post-translational levels[128]. At the translational level, immunoblotting

revealed that alcohol decreased total eNOS in the uterine artery endothelium. Studies have shown diminished eNOS coincides with reduced NO bioavailability and vasodilatory capacity, and that opposite is also true: increased eNOS yields higher levels of NO and vasodilatory capacity[108,109,111,129–131]. This study finding supports our previous claim that a chronic binge alcohol exposure dysregulates the NO pathway component of endothelial-mediated vasodilation in the uterine artery and may account for this vessel's diminished ability to vasodilate.

At the post-translational level, immunoblotting revealed decreased $^{P\text{-}Ser1177}$eNOS in the alcohol group compared to controls. One way that eNOS activity is regulated is by post-translational modification, e.g., phosphorylation, at various sites on amino acid residues such as serine, threonine, and tyrosine[128]. Phosphorylation of the serine 1177 (Ser1177) site is a positive regulator, or activator, of eNOS activity[117,132]. We elected to assess the response of this phosphorylation site to alcohol exposure based on its central role as a positive eNOS regulator: in most instances of eNOS activation, the Ser1177 site is phosphorylated[133]. An array of stimuli and subset of stimuli-specific kinases coordinate phosphorylation of $^{Ser1177}$eNOS, with the most studied stimuli/kinase pair being shear stress and AKT, respectively[117]. In pregnancy, arterial wall shear stress is also the primary stimulus for 1) flow-mediated vasodilation and 2) uterine vascular remodeling[14,49], both of which ensure placental perfusion that is ever-increasing throughout gestation[39,134]. Our immunoblot results indicate a reduction in one type of active, NO-producing eNOS in the maternal uterine artery following chronic binge alcohol exposure, suggesting a reduction in NO bioavailability and vasodilatory capacity of this vessel, which supports our previously discussed findings. While not a conclusive link, a perhaps more interesting connection is the overlap between shear stress and the following physiological endpoints: $^{P\text{-}Ser1177}$eNOS, flow-mediated vasodilation, and vascular remodeling, and their

potential vulnerability to alcohol in the context of the uterine artery and pregnancy. A logical next step would be to assess shear stress and blood flow/flow-mediated vasodilation in this model, both *in vitro* (pressure arteriography) and *in vivo* (ultrasonography) in this critical vessel.

### 6.6. Vascular Study Limitations + Future Directions

Nota bene, alcohol does not always generate similar effects on NO signaling in the endothelium. Chronic, moderate-level alcohol exposure in non-pregnant rats has been shown to increase eNOS and acetylcholine-mediated vasodilation in the thoracic aorta[135]. This study also ascertained that serum NO metabolite levels increased and levels of post-translationally modified eNOS (Ser1177/1179, Thr495/497) were unchanged in the thoracic aorta in response to the same exposure paradigm[135]. However, test subjects treated with a lower alcohol dose/identical duration paradigm did not yield similar "cardioprotective" effects[135]. Therefore, it is essential to apply a caveat to our study: results described above are dependent on alcohol dose/exposure paradigm, vascular location, and pregnancy status.

Several assessments could have been addressed to make the findings from our studies more definitive. Regulation of eNOS via post-translational modification is incredibly complex and currently it is impossible to assess in its entirety. However, evaluation of only P-Ser1177 limits our understanding eNOS regulation in the framework of this study. While the regulatory activity of some phosphorylation sites remains controversial and/or less defined, Threonine 495 (Thr495) is well-characterized as a negative eNOS regulator, meaning phosphorylation here inhibits eNOS activity via calmodulin binding interference[133]. Synchronization between Ser1177 and Thr495 has been well described, where phosphorylation of one often occurs simultaneously

with the dephosphorylation of the other, and vice versa[133,136]. Based on this comportment and our results, it is logical to predict an increase in $^{P\text{-}Thr495}$eNOS would occur in the alcohol group. Testing for this would have provided more conclusive evidence and an expanded understanding of eNOS activity in the uterine artery following alcohol exposure. Additional effort required to testing for $^{P\text{-}Thr495}$eNOS would be negligible.

Evaluation of other post-translational phosphorylation sites known to positively (Serine 633, Tyrosine 81) and negatively (Tyrosine 657) influence eNOS activity[133] would have also generated a more complete description of enzymatic activity in the uterine artery following chronic binge alcohol exposure. To state "if eNOS activity was affected" is myopic and presumptuous, because in addition to other phosphorylation sites with known and ambiguous actions, a host of other reversible post-translational modifications (acylation, *S*-nitrosylation, acetylation, *O*-glycosylation, glutathionylation, protein-protein interactions) ultimately determine the net activity of each eNOS molecule[128,133], none of which have been reported or considered here. Furthermore, while our representative immunostaining identified $^{P\text{-}Ser1177}$eNOS in endothelial cells, a major regulatory determinant of eNOS activity is intracellular localization[128,133,137]. Evidence suggests that eNOS association with the plasma membrane (e.g., caveolae) is associated with enzymatic activation, whereas eNOS positioned at alternate locations (e.g., the cytosol, nucleus, mitochondria, or Golgi apparatus membrane) is associated with enzymatic inhibition or reduced NO production[128,133]. A more thorough immunostaining approach at higher magnification (via confocal or immunoelectron microscopy) would be a likely future direction for detecting eNOS location in relation to various organelles.

Even if subcellular location was not directly assessed by more precise histological methods, other molecular clues can indicate intracellular location, and therefore regulatory

status, of eNOS in the uterine artery endothelium. Fatty acid acylation plays a significant role in eNOS distribution within the cell: eNOS that is myristoylated and palmitoylated has an affinity for binding to caveolae and Golgi membranes, whereas enzyme molecules deficient in this specific post-translational modification are much more likely to be found in the cytosol and unbound to cellular organelles[128]. Detection of this specific post-translational modification via simple molecular validation methods (e.g., PCR, Western blot) is one way to assess eNOS activity and give subcellular localization clues without the use of microscopy[138]. Depending on these outcomes, biotinylation and transgenic models carrying $^{Ser\text{-}1177A/D}$eNOS transgenes (where Ser1177 is replaced with alanine or aspartate) may be useful tools for better understanding eNOS dysfunction in our model of alcohol exposure[138,139].

Another way to expand on our findings would be to assess whether alcohol affected eNOS substrate and cofactor bioavailability in the uterine artery endothelium. eNOS generates NO through conversion of L-arginine (substrate) into L-citrulline and Tetrahydrobiopterin (BH4, cofactor)[81,140–142]. Cellular deficiencies in either arginine or BH4 have been linked with endothelial dysfunction via eNOS uncoupling, which results in generating superoxide anion ($O_2^-$) in place of NO[81,140–143], however these deficiencies have yet to be studied in an analogous model to ours, specifically involving pregnancy and a chronic binge alcohol exposure paradigm. We and others have reported that alcohol disrupts amino acid homeostasis in various tissue types of both the mother and fetus, including the placenta[143–146]. It is possible that in endothelial cells, the amino acid arginine is susceptible to alcohol exposure[147], which could limit the medium for NO synthesis and consequently reduce NO production and NO-mediated vasodilation. Whether alcohol is affecting arginine transport into cells or acting directly on intracellular concentrations of arginine in uterine artery endothelial cells has yet to be determined, but endothelial assessment

of arginine levels could be insightful for understanding alcohol-mediated endothelial vasodilatory deficits. A potential scenario for intracellular arginine depletion would be if alcohol increased the activity of arginase, (an enzyme that competes with eNOS for L-arginine as substrate for converting into L-ornithine and urea) NO synthesis via eNOS could be inhibited[148–150]. Alcohol has been shown to dysregulate arginase activity[151], but investigation of this interaction in the endothelium remains quite limited[152].

Analogs of the L-arginine substrate [such as Nω-nitro-L-arginine methyl ester (L-NAME), $N^G$-Monomethyl-L-arginine acetate (L-NMMA), and $N^G$-nitro-L-arginine (L-NOARG)] are NOS inhibitors[153–156]. In pressure myography studies, these are commonly used to assess the role of eNOS on vasodilatory function. Incubation of vessels in a solution containing these analogs/eNOS inhibitors and then repeating the pressure myograph studies with an Ach dose-response would have theoretically blunted uterine artery vasodilatory response to Ach and would have more resolutely implicated the susceptibility of eNOS to chronic binge alcohol exposure. Omission of this step diminishes the definitiveness of the results presented here.

Tetrahydrobiopterin (BH4) is a necessary cofactor for eNOS catalyzation, and deficiencies result in enzyme uncoupling[141]. Oxidative stress limits BH4 bioavailability via degradation[141], and oxidative stress is also a well-documented response to chronic alcohol exposure[125,157,158]. Post alcohol exposure, BH4 deficiency has been associated with NOS-mediated endothelial dysfunction[159,160], and BH4 supplementation has been shown to mitigate these effects in rat cerebral arteries following a chronic alcohol dosing paradigm[140,142]. Interestingly, in response to the latter chronic alcohol exposure paradigm, eNOS expression in cerebral arterioles was unchanged (in fact, in some similar models, expression has even increased[135,161,162]), but vasodilation to NOS agonists was impaired by alcohol exposure and

restored by nitroglycerine administration as well as by BH4[140,142]. While eNOS expression in our study was decreased, it is still possible BH4 deficiency occurs and has an additive effect on the uterine artery's inability to dilate sufficiently, and testing endothelial BH4 levels, response to BH4 supplementation, as well as determining $NO/O_2^-$ levels could be valuable pieces of the FASD puzzle.

Several other limitations of these studies are worth mentioning as caveats:

1) Since the uterine arteries were harvested 24 hours after the last alcohol administration, we cannot say for certain whether the effects observed here are direct effects of chronic binge alcohol exposure or if they are effects of alcohol withdrawal. Following chronic, heavy alcohol use, a drop in usage can initiate profound disruption to various homeostatic processes. These studies were unable to make this important distinction. It is possible that the observations described here are a direct result of alcohol, alcohol withdrawal, or a combination of these factors.
2) Cells studied *in vitro* do not necessarily behave as they would *in vivo* milieu. Using whole tissue specimens (maternal uterine artery) is more translational than studying cultured cells, but it is possible that the vascular behavior observed in these studies is not entirely representative of what occurs *in vivo*.
3) Several factors were not addressed:
    a. Viability of the endothelium was not determined. Even though this is a more common technique for cell culture experiments, endothelial cell viability can only be implied since it was not validated here. Endothelial cells are relatively delicate compared to other cell types, and colorimetric/fluorometric staining and/or flow cytometry would have been helpful in verifying that the endothelial cells studied

here were still functional post-collection at the time of experimentation[163]. Confirmation of endothelial denudement was also not described.

b. Alcohol's effects on uterine artery hemodynamics *in vivo* were assessed at only one timepoint, 24 hours after the last gavage treatment. Had additional timepoints been assessed, e.g., 1 hour after treatment administration at peak BAC, it would have been possible rule out whether the arterial dysfunction described here was mediated by a withdrawal effect. Additionally, flow studies could have been carried out *ex vivo* with the same pressure myography system used here where the vascular reactivity is measured in response to both controlled translumenal flow and controlled transmural pressure. Not assessing either parameter here was a missed opportunity.

c. Uterine artery pressure response was not assessed in a calcium-free environment. In pressure myography, it is standard procedure following pressure assessment in the presence of circulating calcium-containing buffer to repeat the experiment in calcium-free buffer. This allows us to understand the passive properties of vessel segments in the absence of extracellular calcium and confirms the presence of active vascular tone in the first experiment. Again, carrying this out would have required minimal additional effort and would have bolstered our findings.

d. A true mechanistic study isolates not only the pathway of interest but rules out alternative pathways. There is evidence suggesting PGI2 and EDHF pathway disruption in response to alcohol, and therefore studying these pathways in our model would prove insightful, whether data was positive or negative.

The intricacy of the eNOS regulatory system allows this enzyme to integrate information from various stimuli into effectual, synchronous responses that maintain dynamic homeostatic processes in real-time. Mechanisms underlying the endothelial NO cascade are daunting to assess, especially since aspects of these remain poorly understood even under normal/healthy conditions, and the convolution of alcohol-mediated effects further challenges investigation. However, given the importance to vascular health which in turn supports fetal outcomes, further investigation of the effects of alcohol on NO signaling in the uterine artery during pregnancy is both relevant and warranted, especially since understanding of maternal vascular function in the context of FASD is markedly deficient.

### 6.7. Comment on Brain Investigation

The brain continues to be the central area of emphasis for FASD investigation because the effects of alcohol on fetal brain development are not fully defined. A marked deficit persists in treatment options and intervention strategies for FASD patients in part because of limited understanding of how alcohol damages the brain *in utero*. To further the narrowing of this knowledge gap, we applied our model of chronic prenatal binge alcohol exposure described above to assess brain regions with known vulnerability to developmental alcohol exposure.

In Chapter 4, high-performance liquid chromatography (HPLC) quantified concentrations of 22 amino acids in three fetal brain regions (cerebral cortex, cerebellum, and hippocampus) with well-documented as FASD targets. Major findings from this study were that alcohol profoundly dysregulated amino acid concentrations in the cerebellum and hippocampus, and based on increases in excitatory amino acids (e.g., glutamate, aspartate) in these structures,

inferences for potential excitatory neurotoxicity were discoursed. Despite an extensive catalog discerning the effects of alcohol on the developing brain, excitatory neurotoxicity has been touched on but not adequately pursued[164–167], and alignment of our results with previous works support further investigation into this area. This study also determined that taurine, the most abundant amino acid in the brain, showed acute upsurges in these regions and a theoretical model for factors contributing to this shift was proposed. Since amino acids function not only as neurotransmitters but also coordinate brain development by influencing neuronal proliferation, migration, differentiation and synapse formation and development, it is theoretically possible that the amino acid disturbances reported here reflect root sources of FASD brain pathogenesis.

In Chapter 5, next-generation RNA sequencing (RNA-Seq) was used as an unbiased approach to profile alcohol-mediated changes to hippocampal gene expression and identify novel targets underlying the complex pathophysiology of FASD. Several genes exhibiting dysregulation had been previously identified as having gene-chemical interactions with alcohol (e.g., *Myl2, Phkg2, Neu2, Slc6a13*) or had been linked with developmental and/or neurological deficits (e.g., *Pcsk1, Sspo, Mff*), but had never been directly associated with FASD. The overlap of these genes' associations with FASD here and with FASD-adjacent pathology previously make them likely candidates to pursue in future studies discerning the convoluted nature of FASD pathogenesis. Another notable finding from this study was that genes and canonical pathways related to amino acid synthesis and metabolism exhibited dysregulation following alcohol exposure, which adds value to findings in Chapter 4. Lastly, sex appears to influence alcohol-mediated effects in the hippocampal transcriptome, and this aligns with a substantial body of evidence indicating sex plays a factor in hippocampal outcomes in the context of FASD[168–172].

While these two studies apply novel investigative techniques to brain regions with well-documented PAE vulnerability, the scope of their experimental design limits their capacity to advance knowledge in the FASD field. Some key caveats are worth noting here that were not addressed or did not receive adequate attention in the original articles.

1) We don't know if changes we observed in the brain were a direct effect of alcohol or an effect of alcohol withdrawal. This limitation was also discussed above regarding the uterine artery; however, it is possible that a withdrawal effect could be seen in the brain and not the uterine artery, but it is also likely that withdrawal effects could be much more profound in the developing brain than those occurring in the uterine artery, since effects of alcohol withdrawal in the brain have been linked with profound damage[173–183]. This limitation was mentioned in Chapter 4 regarding amino acid disruption, but the potential for withdrawal effects may also play a role in hippocampal transcriptome dysregulation. Replicating these studies in a model with less potential for withdrawal, such as with a chronic exposure where alcohol was freely administered in the diet would make for an interesting comparison to rule out and/or pinpoint withdrawal effects.
2) The transcriptomic studies should be viewed as speculative because conventional validation for molecular analysis is absent. The argument for bypassing this practice was that next-generation sequencing (NGS) is a much more sensitive than the conventional validation testing methods (e.g., qRT-PCR, in situ hybridization, array-based comparative genomic hybridization, sanger sequencing) and that quality control metrics are met (e.g., read length/depth/quality, strand bias) ensures random error is minimized[184]. Other disadvantages of conventional methods are that they can be used to verify positive data (upregulation) but are less useful regarding negative data (downregulation) and that

discordance between NGS and conventional testing may be attributable to tissue heterogeneity rather than inherent error[184]. Despite these restrictions, conventional molecular validation is recommended standard practice for assessing novel and/or unexpected transcriptome results, especially in pre-clinical exploratory studies with limited application of bioinformatic analyses like the studies described here[184]. In other words, conventional testing is required to substantiate the presence of recurrent gene expression variants in distinct regions of the transcriptome, and omitting this process restricts the scope of the inferences that can be made from study findings.

3) The transcriptome analyses attempt to account for generating "mechanistic insights" in the absence of proteomic comparation. Within a given tissue of interest, the transcriptome represents a snapshot of total genes expressed (or transcribed into RNA from DNA) and pinpoints of a host of mechanisms underlying cellular processes such as of transcriptional and post-transcriptional regulation[185]. The proteome represents a snapshot of the translated protein complement to the transcriptome and gives a functional representation of cellular activity[186]. The proteome is able to assess functionality because it can do several things the transcriptome cannot:
    a. The proteome can predict relative protein abundance within a given tissue or cell type. mRNA expression in the transcriptome does not correspond with protein abundance because the rates of protein synthesis and degradation are dynamic and vary depending on individual mRNAs[186]. Therefore, protein abundance can be many fold-changes higher than its corresponding mRNA transcript expression.
    b. The proteome can detect characteristics that determine protein regulatory activity, such as post-translational modifications, proteolytic cleavage, and whether

proteins are working as a complex[186]. These changes occur post-translationally and therefore cannot be predicted by the transcriptome.

A future comparison of the concordance/discordance between the hippocampal transcriptome with the hippocampal proteome could act as a practical means to corroborate the gene expression findings described herein. More importantly, it would indicate functional changes induced by PAE which would yield much more compelling mechanistic insights and/or candidate genes to more completely discern FASD pathophysiology.

4) The brain analyzed in these studies had not completed fetal development. While this was an intentional design to account for *in utero* dynamics such as placental interactions, it meant harvesting brain tissue at the end of the second trimester-equivalent of brain development and prior to the brain growth spurt. Future comparisons of these study findings with those after completion of "fetal" brain maturation equivalency (roughly postnatal day 10) would be valuable for understanding the extent of gene expression dysregulation that the hippocampus undergoes throughout development. It has been proposed that transient gradients of gene expression in the fetal brain orchestrate patterns of development[187], and under this assumption it is likely that studies in Chapter 5 show more of a snapshot of gene expression changes at one timepoint point rather than net transcription dysregulation in response to PAE.
5) In both amino acid and transcriptome analyses, each structure was assessed as tissue homogenates from entire brain regions. However, specific cell types within each of these structures are known to have distinct responses to PAE[94,188–196]. Had these cell types within brain regions been isolated, it might have offered insight into the where

dysregulation was occurring spatially and might also have been more representative of functional outcomes based on the types of changes observed in specific cell populations. Even if studying individual cell populations exceeded experimental scope, analyzing specific areas within brain regions would have been helpful based on our understanding of the organization of neural networks and cell type distribution within these structures. For example, the dorsal and ventral horn of the hippocampus vary by cell type distribution, receive their respective sensory information from different parts of the brain, and exhibit distinct functional differences[197]. Hippocampal gene expression reflects this polarity indicative of cellular and regional heterogeneity[197]. Homogenizing whole brain regions accounts for entire variations within the hippocampus, but important context is lost for interpreting these results.

Collectively these studies show novel ways in which the fetal cerebral cortex, cerebellum, and hippocampus are susceptible to chronic binge alcohol exposure *in utero*. While these findings suggest novel targets to pursue in future studies discerning FASD pathogenesis, there is an overwhelming lack of direction and context clues due to gaps overlooked in study design. This oversight is unfortunate, given the urgent need for pathophysiological understanding from which to formulate effective treatment strategies for patients afflicted with FASD. Responsible future directions should take steps to better understand excitatory neurotransmitter toxicity and develop a much more in-depth enrichment pathway (IPA) analysis with conventional molecular validation techniques to confidently identify targets with which to advance the FASD field.

FIN

## 6.8. References


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# APPENDIX A

# DYSREGULATION OF UTERINE HEMODYNAMICS *IN VIVO* FOLLOWING CHRONIC BINGE GESTATIONAL ALCOHOL EXPOSURE

## A.1. Introduction

Ultrasound is an ideal imaging approach, as it can be used throughout pregnancy, is non-invasive, and does not emit ionizing radiation to the mother or the developing fetus. Doppler imaging of maternal and fetal vascular resistance indices is an important clinical tool for evaluating high-risk pregnancies with fetal growth restriction outside the context of FASD[1]; however, clinical use of this tool in the context of prenatal alcohol exposure is underutilized. High-resolution ultrasound imaging is showing potential for detecting *in utero* evidence of prenatal alcohol exposure in humans[2-4], but its use assessing vascular sequelae of prenatal alcohol exposure remains limited[5]. One study has identified alcohol-induced alterations to cranially directed fetal blood flow following an acute alcohol exposure[6], however the effects of a chronic binge paradigm in an FASD growth restriction model on uterine blood flow remain unknown. This following data tested the hypothesis that chronic binge alcohol exposure alters uterine artery blood flow and vascular resistance indices in late pregnancy to the maternal uterine artery in an FASD growth restriction model. Assessment of vascular parameters *in utero* via ultra-high frequency ultrasonography will advance our understanding of PAE's maternal health consequences and how these may in turn be predictive of adverse fetal outcomes, as these metrics are currently used clinically to do so.

## A.2. Materials and Methods

### A.2.1. Animals

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996), with approval by the Animal Care and Use Committee at Texas A&M University. Timed pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA), and were housed in a temperature-controlled room (23 ° C) with a 12:12-h light/dark cycle. Rats were assigned to a pair-fed control group (n = 9) or an alcohol treatment group (n = 9) on GD 4. The alcohol animals were acclimatized via a once-daily orogastric gavage of a 4.5 g/kg (22.5% wt/v) alcohol dose from GD 5–10 and progressed to a 6 g/kg alcohol dose from GD 11–19 (28.5% wt/v). Pair fed controls received a daily gavage of maltose dextrin to control for the calories in alcohol and the gavage procedure. All rats were weighed on GD 4 and each pair-fed control rat was matched with a similar weight alcohol rat for the study duration, and these pairs were fed isocalorically as described in chapters 2 and 3. Maternal weight was not different between treatment groups on GD 20, one day after the last gavage administration and when images were obtained.

### A.2.2. Ultrasonography

On GD 20, dams underwent high-frequency ultrasonography with Doppler to assess uterine artery hemodynamics *in vivo*. Animals were sedated briefly for the duration of the imaging procedure with isoflurane (maintained at 2%) in oxygen (maintained at 0.50 L/min) and were secured to a heated imaging platform. Body temperature, heart rate, and respiratory rate were monitored throughout the procedure. The right and left lower abdominal quadrants were shaved and ultrasonic transmission gel (EcoGel 100) was applied to the skin prior to imaging. Ultrasonography was performed using a Vevo 3100 preclinical high-frequency imaging system

with a 40-MHz (MX550D) probe (VisualSonics, Toronto, Canada) to assess the following hemodynamic parameters in the maternal uterine artery: heart rate (bpm), vessel diameter (mm), blood flow velocity (mm/s), blood flow rate (ml/s), pulsatility index, and resistive index. Uterine arteries were identified based on anatomical landmarks and characteristic waveform shape. Images with and without color doppler were obtained in B-mode which recorded 5 seconds of continuous imaging for each capture. After data acquisition, images were analyzed using Vevo LAB software (Fujifilm VisualSonics). Mean maximum velocity was calculated over 3 continuous cardiac cycles within each Doppler wave form. Uterine artery flow rate was determined from the mean peak velocity and the cross-sectional area of each artery. Resistive index is a clinical indicator of vascular resistance in specific vessels and is determined by the following formula:

$$\text{Resistive index} = \frac{\text{Peak systolic velocity} - \text{Minimum diastolic velocity}}{\text{Peak systolic velocity}}$$

Pulsatility index is another clinical indicator of vascular resistance and is determined by the following formula:

$$\text{Pulsatility index} = \frac{\text{Peak systolic velocity} - \text{Minimum diastolic velocity}}{\text{Mean velocity}}$$

Uterine artery blood flow was calculated by determining the vessel cross sectional area from the diameter and multiplying this by mean peak velocity.

### A.2.3. Statistics

Maternal heart rate, velocity time interval (VTI), resistive index (RI), pulsatility index (PI), and uterine artery blood flow were each analyzed by Student's t-test. All data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and are presented as mean ± SEM, with significance established a priori at $p < 0.05$.

## A.3. Results

Maternal mean heart rate was not different ($p = 0.1473$) between the pair-fed control ($n = 8$; 380.07 bpm ± 31.21) and alcohol ($n = 8$; 353.40 bpm ± 43.075) treatment groups (Fig 20). However, maternal uterine artery blood flow ($p = 0.0106$) and VTI ($p = 0.03269$) were significantly reduced in the alcohol group (0.0401 ml/min ± 0.0171; 59.6513 ± 18.0622) compared with controls (0.1227 ml/min ± 0.0679; 74.2614 ± 19.2038) (Fig 20). Indices of vascular resistance (PI, $p = 0.0263$; RI, $p = 0.02899$) were significantly increased in the alcohol group (1.0440 ± 0.2076; 0.6639 ± 0.0667) compared with controls (0.8240 ± 0.1032; 0.5756 ± 0.0463) (Fig 20).

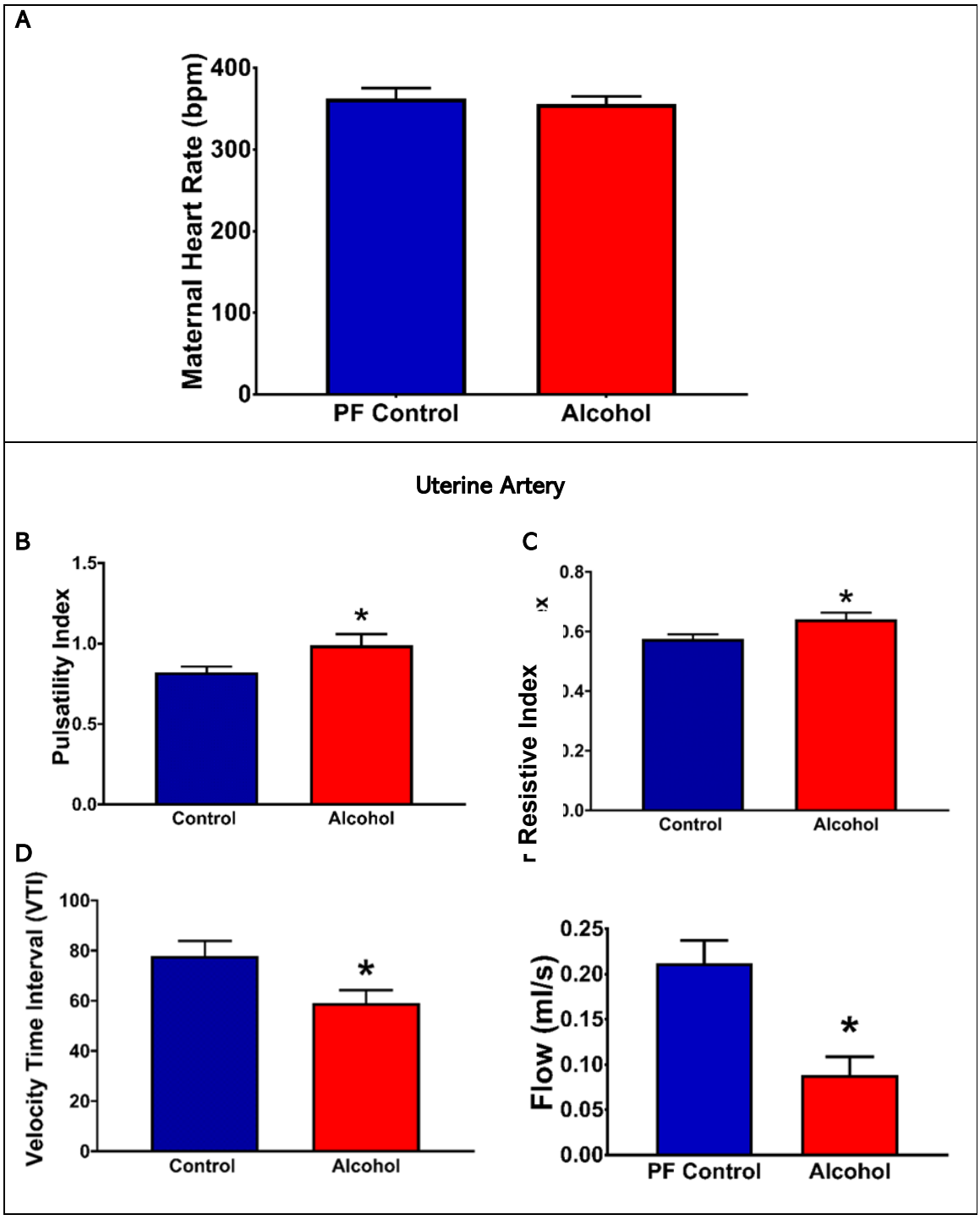


**Figure 20:** *Ultrasound assessment of the maternal uterine artery on gestation day 20*. Maternal heart rate (A) was not different between groups. However, Pulsatility Index (PI) and Resistive index (RI) increased, and Velocity Time Interval (VTI, D) and Flow rate ($mm^3/s$, E) decreased following chronic binge alcohol exposure ($P < 0.05$).

## A.4. Discussion

This data aligns with that in the uterine artery *ex vivo* functional studies described in Chapters 2 and 3 that suggest maternal blood flow to the fetal compartment may be impaired following chronic binge alcohol exposure throughout pregnancy. This data showed that although heart rate was not different between treatment groups, blood flow in the uterine artery to the fetal compartment was diminished following alcohol exposure. In addition to uterine arteries in the alcohol group having reduced cross sectional area (data not shown), these vessels also displayed higher resistance indices, supporting earlier findings that alcohol increases uterine artery resistance to conduction of blood flow. A limitation of this assessment was that data was collected at only one timepoint during pregnancy just prior to parturition. It is possible that these hemodynamic changes do not reflect hemodynamic changes that occur throughout pregnancy in response to alcohol exposure. Since these data were recorded one day after the last alcohol exposure, it is possible the changes here reflect a withdrawal and not a direct effect of alcohol. Additional testing at timepoints throughout pregnancy is warranted to definitively determine the effects on uterine artery hemodynamics during pregnancy following chronic binge alcohol exposure. The hemodynamic changes described here combined with arterial functional changes and fetal growth restriction described previously suggest that alcohol-induced arterial dysfunction in the maternal primary uterine artery which supplies blood (and thus oxygen and nutrients) to the fetal compartment may play a critical role in mediating the wide array of pathological effects comprised in FASD phenotypes.

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