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Nutritional and Oxidative Stress Risk Factors for Fetal Alcohol Spectrum Disorder

By

HARPREET CHIMA
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Approved:

Carl L. Keen, Chair

Roberta R. Holt

Christina Chambers

Committee in Charge

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Abstract

Fetal alcohol spectrum disorder is a leading preventable cause of neurodevelopmental disability. Prenatal alcohol exposure causes these disorders, but maternal nutritional status and oxidative stress responses can modify fetal vulnerability. Animal studies demonstrate that alcohol induces oxidative stress and disrupts maternal micronutrient homeostasis. Human studies linking these physiological disruptions to infant outcomes are lacking.

We analyzed alcohol consumption patterns in pregnant Ukrainian women using growth mixture modeling to identify longitudinal alcohol trajectories. Women who maintained very high drinking levels throughout pregnancy showed elevated oxidative stress markers, lower plasma zinc and copper, and activation of the acute phase response. These biochemical changes correlated with impaired infant growth and neurodevelopment.

The highest alcohol exposure group showed elevated inflammatory markers and compromised micronutrient status. This combination predicted poorer infant outcomes, with effects varying by infant sex. Multivitamin/multimineral supplementation during pregnancy partially improved maternal biochemical markers but did not prevent adverse developmental outcomes.

Our findings provide the first human evidence suggesting that alcohol exposure, acute phase response, and conditional nutrient deficiency may interact to magnify teratogenicity beyond alcohol's direct effects alone. Maternal biochemical status mediates the relationship between prenatal alcohol exposure and developmental outcomes. Preventing FASD may require comprehensive interventions that address both alcohol cessation and maternal physiological support.

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List of Acronyms

1,25(OH)$_2$D 1,25-dihydroxyvitamin D. 23

1CM one-carbon metabolism. 14, 17

5-MTHF 5-methyltetrahydrofolate. 15

AAPH 2,2'-azobis(2-amidinopropane) dihydrochloride. 68

ABAP 2,2'-azo-bis(2-amidinopropane). 100, 163

Abstain Abstained. 109–115, 117–131, 134, 140–142, 162, 167–169, 171, 173, 174, 177, 179–182, 184–186, 188, 190, 193, 194

ACA Active Case Ascertainment. 5, 6

ADH alcohol dehydrogenase. 38, 40, 91, 138

AE alcohol exposed. 95, 160, 202

AIC akaike information criterion. 109, 110, 161

ALD alcoholic liver disease. 15, 19, 27, 32, 36

ALDH aldehyde dehydrogenase. 40

ANOVA analysis of variance. 69, 72, 107, 112

AP-1 Activator Protein-1. 74

APR acute phase response. 2, 92, 136, 137, 158, 191, 194–196, 204, 205, 213

ARBD Alcohol-Related Birth Defects. 3

ARND Alcohol-Related Neurobehavioral Disorder. 3–7

AST/ALT aspartate aminotransferase to alanine aminotransferase. 27

BH Benjamini-Hochberg. 107, 108, 112, 113, 125, 126, 128–132, 142, 143, 165, 166, 168–171

BHT butylated hydroxytoluene. 99

BIC bayesian information criterion. 109, 110, 161

BMI body mass index. 96, 103, 107, 108, 112, 160, 164, 165

BSID-II Bayley Scales of Infant Development. 14, 102, 143, 145, 163, 165, 203

CIFASD Collaborative Initiative on Fetal Alcohol Spectrum Disorders. 95, 141, 143, 159, 193, 194

CNS central nervous system. 32, 158, 193, 196

CP ceruloplasmin. 32, 100, 101, 118, 137, 163, 165, 183, 184, 192, 195–197, 201, 204

CRP C-reactive protein. 27, 182

CuZnSOD CuZn superoxide dismutase. 31, 33, 73, 92, 158, 195

CYP2E1 cytochrome P450 2E1. 38, 40, 91

DMT1 divalent metal transporter 1. 36

DNA Deoxyribonucleic Acid. 12, 13, 17, 31, 39, 73, 91, 93

EDTA ethylenediaminetetraacetic acid. 98

ER endoplasmic reticulum. 74

FAD flavin adenine dinucleotide. 136

FAEEs fatty acid ethyl esters. 38

FAS fetal alcohol syndrome. 3, 4, 6, 7, 20, 27, 90, 141, 157

FASD fetal alcohol spectrum disorder. 1–9, 17, 25, 27, 32, 36, 37, 41–43, 90, 91, 95, 141, 142, 157–159, 201, 204, 205, 213, 215

FBP folate binding protein. 16

FDR False Discovery Rate. 108, 166

G6PD glucose-6-phosphate dehydrogenase. 39

GCL γ-glutamyl-cysteine synthetase. 64, 74, 75

GGC γ-glutamyl-cysteine. 64, 65, 67, 73, 75, 76, 78

GGC-Bis GGC-Bis. 65, 66, 70, 71, 79–87

GGC-HCL GGC-Hydrochloride. 65, 66, 70–72, 75, 79–87

GGC-La GGC-Lipoic acid conjugate. 65, 66, 70–72, 74–76, 79–87

GLM generalized linear model. 108, 124, 127–132, 165–167

GLMM generalized linear mixed model. 107, 114–127, 144

GMM growth mixture model. 106, 143, 145, 161, 202

GPx glutathione peroxidase. 39, 76, 99, 116, 133, 134, 136, 162, 165, 167, 168, 172–175, 197, 198, 200, 201, 204

GRed glutathione reductase. 99, 115, 116, 119, 133–136, 162, 165, 198

GS GSH synthetase. 65

GSH reduced glutathione. 40, 64, 65, 67, 70–72, 74–87, 91, 99, 133–135

GSSG oxidized glutathione. 65, 67, 72, 75, 85, 99, 133, 163

Hb hemoglobin. 98, 99

HIF-1α hypoxia-inducible factor 1α. 32

HighMod High-to-Moderate Sustained. 109–115, 117, 119–123, 125–132, 138, 139, 141, 162, 167–169, 171, 173, 174, 177–182, 184, 186, 189, 190

Hox homeotic. 19

hsCRP high sensitivity C-reactive protein. 101, 116, 117, 120, 121, 137, 163, 165, 182, 192, 196, 197, 200, 201, 204

IACUC Institutional Animal Care and Use Committee. 66

ICP-OES Inductively Coupled Plasma Optical Emission Spectrometry. 100, 163

IL-6 Interleukin-6. 22

KABC-II Kaufman Assessment Battery for Children, Second Edition. 4

LBW low birth weight. 9–11, 26, 31, 34, 35

Low Low Sustained. 109–115, 117, 118, 121–123, 125–128, 130, 131, 140, 162, 168, 169, 171–174, 179–182, 184–188, 199

LT50 time to 50% hemolysis. 69, 72, 76

MDA malondialdehyde. 69, 72, 99, 100, 112–115, 133

MDI Mental Developmental Index. 42, 102, 108, 125–127, 129, 130, 141, 142, 163, 165, 166, 172, 173, 177, 178, 182, 187, 189, 190, 193, 194, 196, 197, 199, 201

MI motivational interviewing. 41, 42

ModDisc Moderate-to-Discontinued/Low Sustained. 109–115, 117, 118, 121–123, 125–131, 139, 141, 142, 162, 168, 169, 171, 173, 175, 179, 180, 182, 183, 185, 186, 188, 190, 199

MPA metaphosphoric acid. 67

MT metallothionein. 25, 27, 28, 31, 33, 73, 92, 137, 194, 195

MVM multivitamin/multimineral. 2, 11, 42, 43, 94–98, 107, 108, 113–132, 134–145, 166, 194, 213–215

MVM PE MVM Prior to Enrollment. 98, 107, 112–123, 127, 129, 131, 135, 136, 138, 143

MVM SE MVM Since Enrollment. 98, 107, 112–123, 126, 128, 130, 131, 138, 140, 142, 143, 145

MZD marginal zinc deficiency. 65, 67, 69–77, 79–87

NAC N-acetylcysteine. 65

NAD+ Nicotinamide adenine dinucleotide (oxidized form). 38

NADH Nicotinamide adenine dinucleotide (reduced form). 38

NADPH nicotinamide adenine dinucleotide phosphate. 99, 116, 119, 133, 162, 163

NF-κB nuclear factor-kappa B. 36, 74

NO nitric oxide. 30, 31

No MVM No MVM Use. 97, 107, 112–123, 126, 128, 130, 131, 133, 143

NOXs NADPH oxidases. 40, 74

Nrf2 Nuclear factor erythroid 2-related factor 2. 74, 134, 135

PAE prenatal alcohol exposure. 1–3, 7, 9, 17, 21, 22, 27, 32, 33, 36, 41–43, 91–94, 108, 132, 135, 136, 138–146, 157–159, 191–205, 213–215

PBS phosphate-buffered saline. 68, 76

PDI Psychomotor Development Index. 102, 108, 127–132, 142, 143, 145, 163, 165, 166, 175, 176, 190, 193, 194, 196, 198, 199

PEG polyethylene glycol. 40

PFAS Partial Fetal Alcohol Syndrome. 3–5

PUFA polyunsaturated fatty acid. 75

RFC reduced folate carrier. 15, 16

RNS reactive nitrogen species. 37

ROS reactive oxygen species. 2, 26, 37, 39, 40, 64, 73, 74, 91, 93, 94, 132, 133, 158, 198, 199

SES socioeconomic status. 96, 108, 160, 165, 166

SOD superoxide dismutase. 26, 30, 33, 39, 40, 76, 91, 98, 99, 114, 115, 117, 118, 133–135, 137, 162, 165, 175, 176, 198

SZD severe zinc deficiency. 77

TBA 2-thiobarbituric acid. 68, 99, 163

TBARS thiobarbituric acid reactive substances. vi, 68, 69, 72, 74, 75, 78, 99, 100, 104, 112–115, 133, 135, 163–165, 178–181, 199

TfR soluble transferrin receptor. 101, 102, 123, 139, 163, 165, 202

TLFB timeline follow-back. 97, 144, 161, 203

TNF-α tumor necrosis factor-alpha. 36, 194, 195

TRAP total radical trapping antioxidant potential. 100, 113, 116, 133–137, 163, 165, 176–178, 198

Trolox 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. 100, 163

TTP α-tocopherol transfer protein. 21, 22

VEGF vascular endothelial growth factor. 30

VHigh Very High Sustained. 109–115, 117–131, 133–139, 141–143, 145, 162, 167–175, 178–184, 186, 188–190, 192–198, 200, 201, 204

VLBW very low birth weight. 31

ZnA zinc adequate. 77

ZnA Control zinc adequate Control. 66, 67, 69–72, 74–77, 79–87

Chapter 1

Maternal Nutrition in Relation to Micronutrient Status, Oxidative Stress, and Fetal Alcohol Spectrum Disorders.

1.1 Introduction

Fetal alcohol spectrum disorder (FASD) represents one of the most common, yet preventable, causes of neurodevelopmental disability worldwide[1,2]. While complete abstinence from alcohol during pregnancy is the only certain prevention strategy, alcohol-exposed pregnancies remain a common and serious public health issue. The clinical outcomes of prenatal alcohol exposure (PAE) are highly variable; not all exposed pregnancies result in FASD, and the severity of effects differs significantly among affected individuals. This variability suggests that the risk is amplified by co-occurring factors[3]. A critical gap in research, therefore, is the identification of modifiable risk factors that could drive new prevention and intervention strategies.

Among these potential factors, maternal nutritional status has emerged as a key determinant of fetal vulnerability. The metabolism of ethanol generates reactive oxygen species (ROS), inducing a state of oxidative stress that can damage the developing fetal brain[4]. Concurrently, alcohol consumption can disrupt the absorption, metabolism, and transport of essential micronutrients required for the body's antioxidant defense systems[3,5,6]. A central mechanism proposed to link these events is an alcohol-induced maternal acute phase response (APR), which can trigger inflammation and lead to a conditioned deficiency of critical nutrients, such as zinc, even with adequate dietary intake[3,7]. While animal models provide strong evidence for these interactions, comprehensive human studies are needed to connect these biochemical pathways to developmental outcomes.

This dissertation, therefore, aims to test a central, twofold hypothesis. First, it posits that the teratogenic effects of PAE are mediated by an alcohol-induced maternal APR, which leads to oxidative stress and conditional nutrient deficiencies. Second, it investigates whether multivitamin/multimineral (MVM) supplementation can ameliorate these maternal biochemical disruptions and, consequently, mitigate the adverse neurodevelopmental outcomes associated with high, sustained PAE. To explore these relationships, this work uses detailed alcohol consumption trajectories to characterize exposure patterns, linking them to maternal biomarkers of oxidative stress, inflammation, and micronutrient status to examine their collective impact on infant growth and neurodevelopment.

This review establishes the foundation for this hypothesis. It begins by outlining the current understanding of FASD diagnosis, prevalence, and public health implications. It then examines the critical role of maternal nutrition in pregnancy, focusing on key micronutrients whose metabolism is disrupted by alcohol. Finally, it explores the mechanisms of alcohol-induced oxidative stress and its impact on fetal development, setting the stage for the research presented in the subsequent chapters.

1.2 FASD Detection and Assessment

The diagnosis and understanding of FASD have evolved significantly, with recent studies providing updated guidelines and insights into the condition's complexity. FASD encompasses a range of disabilities associated with PAE, including fetal alcohol syndrome (FAS), Partial Fetal Alcohol Syndrome (PFAS), Alcohol-Related Neurobehavioral Disorder (ARND), and Alcohol-Related Birth Defects (ARBD). These categories, initially described by the Institute of Medicine in 1996, remain critical for diagnosing and understanding the spectrum of disorders[8].

The updated guidelines by Hoyme et al. (2016) emphasize a multidisciplinary approach to diagnosing FASD, involving a team of experts in pediatrics, clinical genetics, and neuropsychology[8]. The guidelines outline specific diagnostic criteria for each category within FASD, focusing on facial anomalies, growth deficiencies, neurobehavioral impairments, and documented PAE.

Recent research emphasizes the critical role of early diagnosis and intervention in managing FASD. Children with FASD benefit from targeted interventions addressing executive function deficits and self-regulation challenges. The Alert Program for Self-Regulation demonstrates effectiveness in correcting executive function disabilities[9]. This 12-week treatment improves inhibitory control, social cognition, and behavioral regulation, with gains maintained at 6-month follow-up. Neuroimaging evidence shows that the program increases gray matter volume in frontal brain regions[10]. Beyond facility-based approaches, school-based interventions show promise. The GoFAR intervention teaches self-regulation and adaptive strategies through computer games combined with parent training, reducing disruptive behaviors[11,12]. Community-based programs complement these approaches by supporting families. The Families on Track intervention produces medium-to-large effects on child emotion regulation, self-esteem, and anxiety through integrated parent consultation and child skills groups[13,14].

Kalberg et al. (2019) observed that as early as 9 months, infants with FASD exhibited

growth restrictions, such as lower weight, and higher total dysmorphology scores, which include distinctive physical anomalies like specific facial features[15]. Assessments of developmental milestones showed significant differences between children with and without FASD from 18 to 42 months of age. Specifically, infants prenatally exposed to alcohol had more difficulty maintaining attention, faced challenges with visual processing, and scored lower on cognitive assessments, including the Mental Processing Index of the Kaufman Assessment Battery for Children, Second Edition (KABC-II), an instrument that evaluates processing and cognitive abilities.

May et al. (2022) assessed physical measurements to identify FASD in first-grade children[16]. They found that growth deficiencies and characteristic facial features—such as short palpebral fissure length, smooth philtrum, and thin vermilion border of the upper lip—were predictive of FASD. The study supported incorporating features like ptosis, hypoplastic nails, prognathism and altered palmar creases into dysmorphology scoring systems to differentiate children with FASD from their typically developing peers. Although mean total dysmorphology scores did not differentiate between children with ARND and controls, depressed height, weight, head circumference and prognathism did differentiate children with ARND from controls.

The role of physical features in diagnosing FASD has been further explored by Del Campo et al. (2024), who investigated secondary physical features in children with ARND[17]. Their findings suggest that while these features are more frequent in children with FAS, they do not significantly differentiate those with ARND from controls. However, higher dysmorphology scores in ARND were associated with lower cognitive abilities, indicating a correlation between alcohol-related dysmorphology and severity of alcohol-related neurobehavioral impairment. FASD has been proposed to be on a continuum of dysmorphology with maximum expression in FAS, lower in PFAS, and lower in ARND. The May et al. (2022) and Del Campo et al. (2024) study results did not support this proposed view, with their results suggesting that the continuum of dysmorphology is present in those categorized with FAS and PFAS

but may not extend to those classified with ARND[16,17].

Despite advancements in diagnostic criteria, challenges remain in achieving consensus across different diagnostic systems. Discrepancies in diagnostic categories hinder early recognition and comparison of clinical studies[17].

1.3 Prevalence of FASD

FASD prevalence varies across populations and regions in part due to differences in study methodologies and diagnostic criteria. Research using Active Case Ascertainment (ACA) methods, including clinical outreach, recruitment, and diagnostic services in specific populations has documented higher prevalence rates than clinic based studies or surveillance and record reviews[18]. A diagnostic reassessment study conducted in a clinic specializing in the assessment and treatment of high-risk populations showed that 86.5% of children and adolescents with FASD had either been misdiagnosed or were never diagnosed[19]. Early diagnosis correlates strongly with improved long-term outcomes for children with FASD[20]. These findings underscore the significance of both improved study methodologies and early diagnosis in the healthcare sector for children with FASD.

1.3.1 Active Case Ascertainment Studies

ACA studies for FASD require comprehensive approaches to accurately identify and diagnose affected individuals. Casting a wide net for potential cases, rather than focusing solely on severe presentations or cardinal facial features, helps identify cases of PFAS and ARND that might otherwise be missed. Comprehensive maternal risk assessment through empathetic, in-person interviews provides vital contextual information for diagnosis. The timing and framing of consent forms may also influence study participation rates, with appeals to parents' desire for professional insight into their child's development potentially being more effective than random recruitment or appeals to scientific contribution[21].

Over-reliance on the three cardinal facial features in some diagnostic guidelines oversimplifies FASD's complex manifestations and may lead to underdiagnosis. Children with ARND often exhibit growth deficiencies and other minor anomalies, even without cardinal facial features. Traits such as height, weight, head circumference, and total dysmorphology score can differentiate children with ARND from typically-developing children. Despite challenges in conducting ACA studies, including time constraints, budget limitations, and logistical issues, well-established diagnostic protocols and random selection of children can yield reliable prevalence estimates and characteristic profiles of affected individuals and their mothers. Importantly, these estimates are often higher than previously estimated[21,22].

1.3.2 Global Estimates

Popova (2017) conducted a systematic review and meta-analysis to estimate the prevalence of alcohol use during pregnancy and FAS globally[1]. The study found that approximately 10% of women worldwide consume alcohol during pregnancy, with one in every 67 of these women delivering a child with FAS. The global prevalence of FAS was estimated to be about 15 per 10,000 people, which is comparable to the prevalence of Down syndrome in the USA. Lange et al. (2018) expanded on these findings by estimating the global prevalence of FASD among children and youth[2]. Their study reported a prevalence of 7.7 per 1,000 births, with significant regional variations. The European Region exhibited the highest prevalence, while the Eastern Mediterranean Region had the lowest. The study also highlighted that FASD prevalence is substantially higher in specific populations, such as aboriginal communities and children in foster care or children residing in orphanages.

1.3.3 National and Regional Studies

A comprehensive analysis of the prevalence of FASD within special subpopulations in Canada, including children in foster care, correctional facilities, special education, specialized clinical settings, and Aboriginal communities found rates that were 10 to 40 times higher than

those observed in the general population. The authors highlight that children in foster care often face unfavorable circumstances such as parental substance abuse, neglect, and young maternal age, which correlate with increased likelihood of PAE[23]. In a different analysis, Popova et al. (2019)[24] estimated the prevalence of FASD among Canadian elementary school students aged 7 to 9 years in the Greater Toronto Area to be between 2% and 3%.

May et al. conducted extensive research on the prevalence of FASD across various regions in the United States, including a Rocky Mountain city, a Midwestern city, and a southeastern county. Their studies showed FASD prevalence rates ranging from 1.4% to 8.3%[21,25–27]. The authors noted that these prevalence rates likely represent undercounts, as their average consent rate was below 60%, suggesting that families with a history of heavy drinking might have been less likely to consent to participation[21].

In an ongoing study conducted in a rural agricultural region of South Africa, May et al. (2022) reported a total prevalence of FASD at 31%, marking it as the highest documented prevalence globally[28]. This research, part of a series of nine studies in the same region beginning in 1997, involved a cohort of first-grade children in public schools and highlighted the severe impact of FASD in these communities. Over the nine-year period, while the overall FASD rate remained steady, there was a significant shift within the FASD group: the prevalence of FAS decreased, whereas ARND increased. This change may reflect the impact of public health interventions promoting awareness of the risks of alcohol consumption during pregnancy. The widespread dissemination of prevention messages may have led some pregnant women to reduce or stop heavy drinking, lowering the incidence of FAS, the most severe form of FASD. Yet, the increase in ARND suggests that alcohol exposure during pregnancy remains prevalent, and children diagnosed with ARND continue to face substantial challenges.

1.4 Public Health Implications

FASD has far-reaching implications in sectors including healthcare, criminal justice, and social services. Research indicates a strong correlation between FASD and criminal behavior, with individuals affected by the disorder being overrepresented in correctional facilities. For instance, a study in the United States found that 60% of adolescents and adults with FASD had legal issues, although this trend declines in adult populations[29]. In Canada, youth with FASD are 19 times more likely to be incarcerated than their non-FASD counterparts[30]. The disorder's impact on impulse control, executive function, and social skills contributes to these outcomes, highlighting the important role that early diagnosis and intervention can play in reducing contact with the criminal justice system[29].

The economic burden of FASD is substantial, affecting individuals, families, and society at large. Healthcare costs for children with FASD are significantly higher than for those without the disorder, with outpatient services utilization being six times higher and emergency room visits twice as frequent. Moreover, the financial strain extends beyond direct medical expenses, as individuals with FASD frequently need ongoing and multidimensional services to manage the disorder's evolving impact[31]. These services include special education, residential care, and social services. Additionally, the disorder's impact on productivity, both for the affected individuals and their caregivers, further exacerbates the issue[30]. In addition to healthcare costs, FASD incurs significant costs in the criminal justice system.

The social stigma and intergenerational impacts of FASD further complicate the challenges faced by affected individuals and their families. FASD is deeply rooted in social issues, with intergenerational impacts that exacerbate the adversity experienced across people's lifespan. Individuals with FASD often encounter high rates of co-occurring health concerns, such as mental health problems and substance use, which are compounded by environmental stressors and experiences of trauma. These factors contribute to a heightened vulnerability to stress and risky behaviors, including alcohol-exposed pregnancies, which perpetuate the cycle of FASD across generations. The complex needs associated with FASD present signifi-

cant challenges for caregivers, leading to feelings of distress, isolation, and grief. Caregivers frequently report being under-supported and misunderstood by service providers, which adds to their burden. As individuals with FASD transition to adulthood, the limited availability of services necessitates that caregivers continue to play a long-standing role in assisting and advocating for their family members, underscoring the need for comprehensive support systems[32].

Given the profound and lasting impacts of FASD, research has increasingly focused on identifying modifiable risk factors that influence fetal vulnerability to alcohol. While PAE is a cause, it is clear that not all alcohol-exposed pregnancies result in FASD, suggesting that other biological and environmental factors play a critical role. Among these, maternal nutritional status has emerged as a key determinant of fetal outcomes. The following sections will explore the foundational role of maternal nutrition, first by establishing the general link between diet and pregnancy outcomes, and then by delving into the specific micronutrient interactions that may mediate the teratogenic effects of alcohol.

1.5 The Links Between Maternal Diet and Pregnancy Outcomes

Maternal dietary patterns during pregnancy are closely associated with pregnancy outcomes. Poor nutritional status is linked to adverse outcomes such as low birth weight (LBW), preterm birth, and increased risk of neonatal complications[33,34]. Among the effects of maternal nutrition, this review considers its influence on risk of FASD.

1.5.1 Historical Evidence from Famine Studies

Maternal nutrition plays a pivotal role in determining pregnancy outcomes and fetal development, impacting factors such as fetal growth, birth weight, and long-term health[33,35]. Historical famine studies provide compelling evidence of this. During World War II, research

conducted of famine events in Netherlands, Leningrad, and Germany highlighted the severe consequences of maternal malnutrition due to acute food shortages. In the Dutch famine of 1944-45, maternal caloric intake dropped as low as 590 calories per day, leading to significant maternal weight loss and a reduction in mean birth weight by approximately 300 grams. The most pronounced effects were observed in infants conceived before the famine but born during it, with perinatal mortality rates increasing sixfold[36].

Similarly, the Leningrad siege from 1941 to 1943 resulted in drastic reductions in food intake, with bread rations falling to 250 grams per day for manual workers. This led to a decrease in mean birth weight by nearly 550 grams and a substantial increase in perinatal mortality, with over a quarter of babies dying during the peak of famine. After the end of World War II in Germany, although the food shortage was less severe, birth weights still decreased by 170 to 227 grams during the worst deprivation periods. Overall, these famine studies demonstrate that acute severe maternal malnutrition can adversely affect fetal birth weight and increase the risk of perinatal mortality[36].

1.5.2 Evidence from Nutrient Supplementation Trials

Balanced protein-energy supplementation during pregnancy has been demonstrated to significantly reduce the risk of LBW, small-for-gestational-age births, and stillbirth, particularly in undernourished women[36]. Furthermore, a number of essential nutrients are critical for neurodevelopment, with an increased risk of developmental defects with reduced maternal status. For example, periconceptional folic acid supplementation is crucial in significantly reducing the risk of neural tube defects, underscoring the role of specific nutrients in fetal development[37]. The essential nutrient choline is critical for fetal brain development and potential promoter of offspring neurodevelopmental health. Choline can be produced endogenously by the liver but in insufficient quantities to meet the Dietary Recommended Intake of 450 mg/day. Most pregnant women in the United States fail to meet the recommended intake of 450 mg/day[38,39]. Studies indicate that choline supplementation can improve cogni-

tive functioning in animal models of Down Syndrome[40] and reduce anxiety-related behaviors in offspring exposed to prenatal stress[41]. Kable et al. (2015) observed improved encoding and memory of environmental events in infants whose mothers received choline supplementation during pregnancy, whether the mother drank alcohol or not[42]. The authors cautioned that these findings should be interpreted carefully, as several study limitations and potential confounding factors exist, including the absence of a choline-only supplementation group, possible interactions between choline and MVM supplements, unique characteristics of the Ukrainian sample (such as lack of folic acid food fortification and exposure to environmental effects from Chernobyl), and limited generalizability to other populations.

Research on multiple micronutrient supplementation during pregnancy, particularly in low- and middle-income countries, has shown promising effects on birth outcomes. Meta-analyses indicate that such supplementation, when compared to iron-folic acid supplementation, more effectively reduces the risk of LBW and small-for-gestational-age births, while also increasing mean birth weight[43–45]. However, the impact of multiple micronutrient supplementation on preterm birth, stillbirth, and maternal or neonatal mortality remains inconclusive[45,46].

Despite these promising results for birth outcomes, many long-term follow-up studies do not find additional benefits for child survival, growth, or cognitive development beyond infancy when compared to iron and folic acid alone[47]. Additionally, systematic reviews have identified an increased risk of neonatal death with multiple micronutrient supplementation in subgroups that began the intervention after the first or second trimester. The reasons for these findings are not entirely clear, but they may be linked to factors such as low maternal education, which correlates with a higher likelihood of home births, limited access to healthcare, and reduced neonatal care-seeking behavior[43].

By prioritizing the improvement of maternal nutrition both before and during pregnancy, maternal and infant health outcomes can be improved. This approach not only supports optimal fetal development but also has the potential to mitigate the effects of exogenous

toxins, such as alcohol.

1.6 Key Micronutrients: Interactions with Alcohol and Fetal Development

Having established the broad importance of maternal nutrition, this review will now examine specific micronutrients whose metabolism is disrupted by alcohol and are critical for neurodevelopment. Deficiencies in these nutrients can exacerbate alcohol's teratogenic effects.

1.6.1 Folate and Choline

Folate and choline are critical for neural tube closure, brain development, and gene expression regulation during pregnancy. Deficiencies in these nutrients can lead to neural tube defects and impaired cognitive development in the fetus, underscoring their essential roles in maternal and fetal health.

Folate and Choline and Fetal Development

Pregnant women are advised to consume between 400 and 800 μg of folate daily to support the growth of maternal, placental, and fetal tissues. The placenta plays a crucial role in extracting, concentrating, and transporting folic acid into fetal circulation, resulting in fetal folate levels that are two to four times higher than maternal levels[48]. Folate is essential for cellular proliferation, neural stem cell differentiation, and growth, as well as for reducing apoptosis-induced cell death and maintaining Deoxyribonucleic Acid (DNA) synthesis in the fetus. A deficiency in folate during pregnancy can lead to cell abnormalities and cell death, particularly in rapidly proliferating cells, and predispose the fetus to neural tube defects. Animal research has demonstrated that maternal folate intake significantly impacts fetal brain progenitor cells during late gestation. Offspring of folate-deficient mice exhibit fewer

replicating neural progenitor cells in various brain regions and increased apoptotic cells in the fetal septum and hippocampus[48].

Choline intake is recommended to increase from 425 mg/day in nonpregnant women to 450 mg/day during pregnancy, as it is transferred from the mother to the fetus, with concentrations in the amniotic fluid and fetal plasma significantly higher than in maternal blood[48]. Choline, similar to folate, is crucial for brain development, including neural tube closure, hippocampal development, and the formation of neural pathways and gene expression involved in memory processes. It is a component of sphingomyelin, which facilitates efficient nerve signal transmission, and as a methyl donor in DNA methylation, it regulates gene expression involved in synaptic plasticity, learning, and memory. Choline also plays a role in homocysteine regulation, cell membrane phospholipid composition, cell signaling lipid biosynthesis, and is a constituent of the neurotransmitter acetylcholine, which influences various processes in the developing brain, supports hippocampal development, and regulates multiple processes including attention, learning, and memory. Animal studies have shown that gestational choline levels significantly affect fetal brain development, with maternal choline deficiency leading to decreased progenitor cell proliferation and neurogenesis, and increased apoptosis in the fetal hippocampus, while supplementation enhances hippocampal neurogenesis[48].

Some studies have explored the combined effects of folate and choline on fetal brain development. Craciunescu, Johnson, and Zeisel (2010) found that choline supplementation could potentially moderate the impact of dietary folate availability on neural progenitor cells in the fetal forebrain during late gestation[49]. Specifically, they reported that choline supplementation in folate-deficient mice resulted in a mitosis rate and hippocampal apoptosis rate that were intermediate between control mice and folate-deficient mice. As both folate and choline are methyl-donor nutrients influencing neurogenesis and apoptosis, choline supplementation might alleviate the effects of folate deficiency on brain development, potentially through epigenetic mechanisms such as gene methylation[48]. Similarly, in mice with a mutation that

disrupts folate metabolism, mild choline deficiency altered maternal one-carbon metabolism (1CM) and increased the occurrence of developmental defects[50]. Hammoud et al. (2021) further investigated the interaction between dietary folic acid and choline during pregnancy[51]. They found that varying levels of these nutrients in pregnant Wistar rats altered offspring 1CM and hypothalamic gene expression, leading to changes in food intake regulation and metabolic parameters in male offspring.

Folate and Choline and Offspring Cognitive Development

Research indicates a significant relationship between maternal folate intake during pregnancy and offspring brain development and cognition. Animal studies have shown that maternal folate deficiency is linked to short-term memory impairment and increased anxiety-related behavior in offspring[50,52]. Human studies have demonstrated positive associations between maternal folic acid supplementation and children's cognitive outcomes[53,54]. For instance, a study by Chatzi et al. (2012) found that children of mothers who took daily folic acid supplements of 5000 μg or more throughout pregnancy scored higher in receptive and expressive communication on the Bayley Scales of Infant Development (BSID-II) compared to controls[55].

In rodent models, increased maternal choline intake during pregnancy has been associated with enhanced cognitive abilities in offspring[56]. Cognitive benefits observed in offspring include enhanced spatial memory, increased memory capacity, reduced proactive interference, and heightened object exploration[57–59].

Human studies have also observed enhanced cognitive function in offspring associated with increased maternal choline intake during pregnancy, using similar methods to those investigating maternal folate levels. One study found that infants born to mothers supplemented with 930 mg of choline chloride per day exhibited improved information processing speed, despite both groups having choline levels above the adequate intake for pregnant women[60]. Another study associated prenatal choline intake with memory scores in seven-

year-old children, but not with intelligence scores, despite the mothers' choline intake being below the recommended level[61]. Furthermore, maternal plasma choline at 16 weeks gestation was positively associated with infant cognitive development at 18 months[62]. However, a study by Signore et al. (2008) found no association between maternal gestational free and total serum choline concentrations and children's visuospatial processing or memory at five years[63].

Alcohol's Impact on Folate and Choline Metabolism

Chronic alcoholism is associated with folate deficiency, primarily due to dietary insufficiency and disruptions in folate homeostasis. These disruptions include reduced absorption in the small intestine, decreased hepatic uptake and storage, and increased urinary excretion of folate[64]. The primary mechanism by which ethanol induces folate deficiency is through intestinal malabsorption[65]. *In vitro* studies have shown that chronic alcohol consumption leads to decreased expression of the intestinal reduced folate carrier (RFC) and folic acid transport in micropigs[66,67]. Similarly, chronic ethanol-fed rats exhibit reduced expression of RFC and proton-coupled folate transporter in the intestinal mucosa, leading to decreased folate transport across isolated intestinal membranes[68]. This deregulation in the intestinal folate uptake system likely contributes to reduced folate levels during chronic alcoholism[65].

Hepatic folate metabolism involves the transport of circulating 5-methyltetrahydrofolate (5-MTHF) across the hepatic membrane, its binding to intracellular proteins, and the synthesis of polyglutamyl folates, which are then secreted through the biliary ductules or hepatic vein. Disruptions in this cycle can lead to folate deficiency[65]. Clinical studies have demonstrated a two-fold acceleration in the development of folate deficiency with megaloblastic anemia in patients with alcoholic liver disease (ALD) on a folate-depleted diet compared to healthy adults on a similar diet[69]. This may be partly due to decreased hepatic uptake of 5-MTHF, as observed in alcohol-fed monkeys[64,70].

Chronic alcoholism may also lead to increased urinary folate loss, as evidenced in ethanol-

fed rats[64,71]. Recent studies suggest that this increased folate excretion results from impaired folate reabsorption in the kidneys, linked to decreased expression of key folate transporters in renal tissue, namely the RFC and folate binding protein (FBP). The downregulation of RFC and FBP reduces folate uptake across renal absorptive surfaces, diminishing folate conservation and contributing to folate deficiency observed in alcoholism[72].

Acute alcohol exposure impacts folate levels by affecting both serum folate levels and tissue folate uptake. A study involving human volunteers demonstrated a decrease in serum folate levels within eight hours of oral or intravenous ethanol administration, with levels rapidly normalizing after cessation of alcohol exposure[64,69].

While there is no evidence that alcohol directly disrupts the absorption of dietary choline, alcohol consumption may precipitate a choline deficiency by increasing choline demand. This reduction in choline availability can have diverse health implications, including neurological disorders and liver disease. For a developing fetus, choline is essential as a precursor to membrane components and neurotransmitters, and it plays a role in gene expression regulation through its function as a methyl donor[73].

Initially, it was postulated that alcohol indirectly impacts the liver as a complication of malnutrition associated with chronic alcohol consumption by depriving the liver of lipotropic substances[74]. However, research from the 1940s and 1950s demonstrated that choline supplementation could mitigate the fibrosis induced by alcohol consumption, suggesting that alcohol might have a more direct effect on the liver[74–76].

Expanding on previous research that documented increases in choline oxidation by liver mitochondria in animals fed with ethanol[77], a study examining the activity of liver choline dehydrogenase in a rat model noted a progressive increase in activity alongside alcohol intake. After prolonged alcohol consumption (105 days), hepatic choline dehydrogenase activity was markedly higher compared to control rats[78]. This suggests that increased oxidation of choline could account for the heightened dietary requirements and deficiencies associated with long-term alcohol consumption.

Recent studies suggest that alcohol-induced choline deficiency could be due to an increased demand for choline in two ways. The enhanced export of hepatic lipids, generated through the metabolism of alcohol via the formation of very low-density lipoproteins, requires choline[73,79]. Additionally, alcohol-induced folate deficiency may lead to a secondary deficiency when choline is utilized to provide methyl groups for the 1CM pathway[73].

PAE: Interactions with Folate and Choline in Fetal Development

Research has examined the interactions between PAE and maternal dietary supplementation with folate and choline, focusing on their effects on fetal brain development. For instance, studies have shown that folic acid supplementation in pregnant rats exposed to alcohol can reduce PAE-induced neuroapoptosis in neonates[80]. Similarly, concurrent folic acid supplementation in alcohol-exposed mice has been found to mitigate the effects of PAE on motor activity and anxiety[81].

Choline supplementation has also shown promise in ameliorating the effects of PAE. In animal studies, maternal choline status has been found to mediate the impact of alcohol on neurobehavioral outcomes such as deficits in working memory[82,83]. In mice fed a liquid diet containing ethanol, choline supplementation prevented DNA hypomethylation and normalized gene expression in the neocortex[84].

Human studies have corroborated these findings, demonstrating that prenatal choline supplementation in pregnant women consuming alcohol can improve infant development. A trial involving pregnant women with heavy alcohol use found that choline supplementation improved infant eye blink conditioning and visual recognition memory, responses often impaired in infants with FASD[85]. A later study indicated that choline supplementation mitigated PAE-induced reductions in infant brain volume and was associated with improved memory[86]. However, in another study, while multivitamin supplementation improved cognitive outcomes in infants exposed to PAE, additional choline supplementation did not provide further benefits[87].

1.6.2 Vitamin A

Vitamin A plays a crucial role in various physiological processes, including immunity, neurodevelopment, and embryonic development. Its significance becomes particularly evident during pregnancy, where its deficiency can lead to adverse outcomes for both the mother and the fetus.

Vitamin A and Pregnancy

The decline in serum retinol levels during pregnancy, especially in the third trimester, is well-documented[88]. This decline is attributed to hemodilution due to expanding blood volume and inadequate nutritional status[89]. The decline is more pronounced in women from low socioeconomic communities, where poor nutritional status and low dietary intake of vitamin A contribute significantly. In deficient populations, vitamin A supplementation during pregnancy has been shown to increase serum retinol levels, and to reduce night blindness, nutritional anemia, and maternal postpartum infections[89]. Furthermore, a substantial reduction in maternal mortality has been observed following vitamin A or β-carotene supplementation in malnourished, vitamin A-deficient women in rural Nepal[90].

Vitamin A and Embryonic Development

Vitamin A is crucial for embryonic development, as demonstrated by studies on pigs and rats born to mothers with vitamin A deficiency. In pigs, maternal vitamin A deficiency led to a variety of defects in offspring, including blindness, cleft palate, and kidney malformations, with some sows failing to show estrous symptoms and others resorbing all fetuses[91]. Similarly, research on rats has shown that approximately 75% of offspring from vitamin A-deficient mothers exhibited developmental anomalies, particularly in the eyes, genitourinary tract, diaphragm, heart, and major arteries[92]. The essential role of vitamin A in embryonic development is further supported by its involvement in critical processes such as cellular differentiation and gene transcription[93].

Vitamin A and its retinoid metabolites play a crucial role in neurodevelopmental pathways, influencing cognition through cellular differentiation and gene transcription during embryogenesis, growth, and differentiation[94]. It impacts the expression of homeotic (Hox) genes essential in neural tube formation[95] and is vital for the functioning of the hippocampus, which underlies learning and memory[96]. Gestational vitamin A deficiency may indirectly affect offspring cognition by impairing sensorineural hearing[97]. Some other studies have shown positive associations between maternal vitamin A and offspring motor scores or language quotients[98], while others have shown no significant impact on intellectual or motor function of older children[99,100].

Impact of Alcohol Consumption on Vitamin A Metabolism

Vitamin A deficiency is prevalent among individuals who chronically consume alcohol, as alcohol interferes with the absorption and metabolism of vitamin A[101]. Alcoholics often exhibit decreased plasma retinol levels and plasma retinol-binding protein concentrations, a phenomenon first highlighted in a landmark study by Leo and Lieber (1982)[102]. This study demonstrated a significant reduction in hepatic vitamin A content, correlating with the severity of ALD. The study found that alcoholics with advanced liver disease, such as hepatitis and cirrhosis, showed a marked decrease in hepatic retinoid stores, even when plasma retinol levels appeared normal. This underscores the potential for significant hepatic retinoid depletion in alcoholics, which may not be immediately evident through plasma retinol measurements alone[101].

Research involving alcohol-fed baboons and rodents has established that alcohol can deplete hepatic retinoid stores independent of dietary vitamin A intake and absorption. In controlled studies, chronic ethanol consumption led to a significant decrease in hepatic vitamin A levels in baboons and rats, despite a nutritionally adequate diet. For instance, baboons fed a diet with 50% of total energy as ethanol developed fatty liver and experienced a 59% decrease in hepatic vitamin A levels after four months, progressing to a 95% decrease

with fibrosis or cirrhosis after 24-84 months[103]. Similarly, rats fed ethanol showed a 42% decrease in hepatic vitamin A levels after three weeks, with continued decline up to nine weeks, even when dietary vitamin A was increased fivefold[103]. This suggests that chronic alcohol consumption accelerates the mobilization and catabolism of vitamin A from the liver, leading to a progressive depletion of hepatic retinoid stores. The depletion rate of vitamin A from endogenous hepatic storage was observed to be 2.5 times faster in ethanol-fed rats than in controls[103,104].

Ethanol and Fetal Development

Ethanol consumption during pregnancy has been shown to lead to both increased and decreased levels of retinol and retinyl palmitate in fetal tissues[105,106]. The study by Grummer and Zachman (1990) investigated this phenomenon in pregnant rats, showed that ethanol ingestion led to fewer fetuses per pregnancy, increased resorptions, and a higher incidence of fetal abnormalities[105]. Their study found that ethanol exposure resulted in elevated vitamin A concentrations in maternal lung and kidney tissues. In fetuses, free retinol levels in the liver were higher at day 17 of gestation compared to control fetuses, but by day 21, retinyl palmitate levels in the liver, lung, and kidney were reduced, indicating disrupted vitamin A metabolism due to maternal ethanol consumption.

Building on these findings, Dejonge and Zachman (1995) explored the potential link between ethanol exposure in pregnant rats, vitamin A metabolism, and cardiac defects in fetal alcohol syndrome[106]. Their research demonstrated that ethanol exposure increased fetal retinol and retinyl palmitate levels while decreasing retinoic acid levels in fetal hearts. These findings support the hypothesis that ethanol-induced changes in vitamin A metabolism may contribute to the pathogenesis of FAS.

The disruption of vitamin A homeostasis has been implicated in developmental brain anomalies, including congenital malformations such as hydrocephalus, where excess cerebrospinal fluid builds up in the ventricles of the brain. Goez, Scott, and Hasal (2011)

reported a case of a newborn exposed to alcohol throughout pregnancy, presenting with low serum vitamin A and hydrocephalus[107]. They proposed that the disruption of fetal vitamin A homeostasis might compromise the activity of the floor plate, a structure crucial for neural tube development. This case was the first to suggest a possible link between PAE, decreased serum vitamin A levels, and congenital brain anomalies in humans, highlighting the need for further research and potential vitamin A screening and supplementation for newborns of mothers who consumed alcohol during pregnancy.

Research has also demonstrated that retinoic acid, a metabolite of vitamin A, can counteract some of the harmful effects of ethanol on fetal development. Twal and Zile (1997) found that ethanol treatment in quail embryos led to cardiovascular abnormalities similar to those caused by vitamin A deficiency[108]. However, these abnormalities were prevented by the addition of retinoic acid, suggesting a protective role.

1.6.3 Vitamin E

The Role of Vitamin E in Pregnancy and Fetal Development

Vitamin E, particularly in the form of α-tocopherol, is crucial for the health and development of both the mother and fetus during pregnancy. It plays a significant role in fertility, implantation, birth weight, and early neural development[109–111]. Maternal serum levels of vitamin E increase during gestation[109]. This increase is thought to counteract the oxidative stress associated with pregnancy, potentially reducing oxidative injury to both the mother and fetus[109,112]. This is particularly relevant in conditions like preeclampsia, characterized by high blood pressure and linked to oxidative stress[110].

Research using zebrafish models indicates that α-tocopherol deficiency causes developmental delays and increased mortality after fertilization. Specifically, the knockdown of α-tocopherol transfer protein (TTP) expression results in severe malformations of the head and eyes, underscoring TTP's essential role in embryogenesis by facilitating the delivery of α-tocopherol to critical sites within the developing central nervous system[113]. These find-

ings suggest that adequate vitamin E, mediated by TTP, is necessary for proper vertebrate embryonic development, implying a similar requirement in humans[111].

Vitamin E and Alcohol Consumption

Heavy alcohol consumption has been shown to significantly reduce plasma levels of α-tocopherol as observed in a study by Bjørneboe et al. (1987), which reported a 37% decrease in α-tocopherol levels among alcoholics compared to controls[114]. González-Reimers et al. (2014) further identified lower tocopherol levels in alcoholics, particularly those with cerebellar atrophy, suggesting a connection between vitamin E deficiency, oxidative stress, and brain alterations[115]. Their study also found an inverse relationship between vitamin E levels and proinflammatory cytokines, such as Interleukin-6 (IL-6), indicating that inflammation from alcohol consumption could deplete vitamin E.

Animal studies support these findings, with research on guinea pigs by Hidiroglou and Madere (2000) demonstrating that alcohol exposure reduces hepatic vitamin E levels when expressed as per milligram total lipid[116]. PAE has been shown to harm fetal development, but vitamin E may offer protective effects. Wentzel, Rydberg, and Eriksson (2006) found that vitamin E supplementation in pregnant rats exposed to ethanol normalized fetal development and reduced oxidative stress markers[117]. Similarly, Shirpoor et al. (2015) demonstrated that vitamin E administration alongside ethanol reduced heart abnormalities and oxidative stress in rat offspring[118]. Sánchez-Campillo et al. (2023) found that maternal vitamin E levels during pregnancy were associated with higher antioxidant status in both mothers and newborns, suggesting a potential influence on newborn antioxidant status[119].

1.6.4 Vitamin D

Recent research highlights the potential influence of maternal vitamin D status during pregnancy on various maternal and fetal outcomes. Despite observational studies supporting these associations, evidence from randomized controlled trials remains inconsistent[120].

The most conclusive evidence supports vitamin D supplementation for preventing neonatal hypocalcemia[121].

Vitamin D Metabolism and Pregnancy

Vitamin D metabolism undergoes significant changes during pregnancy, with circulating levels of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) increasing substantially. During this period, high concentrations of $1,25(OH)_2D$ indicate a decoupling of vitamin D from its traditional role in calcium metabolism. Instead, vitamin D functions primarily as an immunomodulator during pregnancy, promoting maternal immune tolerance to the semi-allogeneic fetus and supporting the developing fetal immune system[122]. Current recommendations emphasize the importance of monitoring and treating vitamin D deficiency during pregnancy, although optimal levels remain undetermined[123]. The role of vitamin D in pregnancy is crucial, as it supports fetal growth, fetal skeletal development, and immune maturation[124].

Maternal Vitamin D and Fetal and Offspring Outcomes

Maternal vitamin D deficiency during pregnancy is linked to adverse outcomes for both the mother and fetus. Studies have associated low maternal vitamin D status with increased risks of preeclampsia and gestational diabetes[124]. Fetal consequences include impaired lung development, neurocognitive difficulties, and reduced bone mass[125]. Low maternal vitamin D levels are also associated with an increased risk of small-for-gestational-age infants[126]. Animal studies have shown that vitamin D deficiency can alter fetal brain development, affecting speech and language development and dopamine synthesis[127]. Limited evidence suggests potential links to autism spectrum disorder, attention-deficit/hyperactivity disorder, and schizophrenia[128].

Alcohol Consumption and Vitamin D

Studies have shown that alcohol intake can alter the expression of key enzymes involved in vitamin D metabolism, such as CYP27B1 and CYP24A1, which are essential for maintaining vitamin D homeostasis[129,130]. These enzymatic changes can lead to disruptions in the concentrations of vitamin D metabolites, potentially affecting the balance necessary for optimal fetal development[129,131]. Recent research highlights that pregnant women who consume alcohol have significantly lower vitamin D levels, during seasons with low sun availability compared to low/no alcohol exposed pregnant women, increasing the risk of vitamin D deficiency and potentially adverse pregnancy outcomes[132].

1.6.5 Zinc

Effect of Pregnancy on Maternal Zinc

Pregnancy increases zinc demand to support fetal growth and development. The additional zinc requirement during human gestation is approximately 1540 μmol, or less than 100 mg, based on the total weight of pregnancy tissues gained and their zinc concentration. The fetus uses 57% of this additional zinc, while uterine muscle uses 24%[133]. To meet these increased needs, the body adjusts zinc homeostasis. Pregnant women consume an average of 25-28 mg/day of zinc[134], with little evidence of increased intake during pregnancy. Animal studies suggest that homeostatic adjustments in zinc utilization, such as changes in intestinal zinc absorption[135], may be the primary mechanism for meeting additional reproductive zinc demands. Maintaining normal maternal serum zinc concentrations is crucial for sufficient zinc transfer to the fetus[133].

Several conditions can interfere with zinc absorption and fetal zinc transport, potentially impacting maternal and fetal health. High intake of diets rich in phytate, a potent zinc absorption inhibitor, is common among women in developing countries and may lead to secondary zinc deficiency. High intake of supplemental iron may compete with zinc in

the absorption process, potentially depressing plasma zinc concentrations[133]. Alcohol abuse during pregnancy may alter zinc metabolism, potentially contributing to FASD and developmental defects. A maternal acute phase-induced response precipitated by physiological stressors, inflammation, or toxicant exposure such as alcohol has been shown to induce secondary zinc deficiency in pregnant animals by stimulating metallothionein (MT) synthesis, a cysteine-rich binding protein that sequesters zinc in the liver, leading to decreased plasma zinc concentrations[3,136].

Effect of Maternal Zinc on Fetal Development and Offspring Outcomes

Zinc plays a crucial role in fetal development due to its involvement in various biological processes, including cellular growth, differentiation, and the development of the central nervous system. Research has consistently demonstrated the importance of zinc in fetal development, with significant consequences of zinc deficiency observed in both animal and human studies.

Early animal studies laid the foundation for understanding zinc's role in development. Todd, Elvehjem, and Hart (1933) first demonstrated the essentiality of zinc using rat models, observing severe growth retardation in zinc-deficient rats[137]. Hurley and Swenerton (1966) later showed that rats fed a zinc-deficient diet exhibited not only severe growth retardation but also other deficiency symptoms, such as lack of fur growth and abnormal appearance, which were reversed upon zinc supplementation[138]. Furthermore, female rats subjected to mild zinc deficiency produced offspring with a wide range of congenital malformations affecting multiple organ systems, including skeletal deformities and heart defects.

National surveys from 20 low- and middle-income countries report that zinc deficiency is a widespread public health concern, with the majority of countries showing a prevalence of low plasma zinc concentration exceeding 20% in both children and women of reproductive age, suggesting the need for preventative zinc intervention strategies[139]. This deficiency can lead to severe consequences, including neural tube defects, growth retardation, and various developmental issues. Even marginal zinc deficiency, which is more common, has been linked

to neurological deficits, behavioral abnormalities, and cognitive impairments in both animal models and human studies[140].

Some studies have reported beneficial effects of zinc supplementation during pregnancy such as reducing the risk of preterm birth and LBW. However, evidence for other outcomes remains mixed due to variations in duration, dose, and pregnancy state at the start of zinc supplementation[141,142]. While several research studies found no significant impact on birth weight or other fetal growth measurements[143,144], other studies noted reduced risks of preterm birth, stillbirth, and early neonatal morbidity[142,145].

Zinc deficiency causes broader physiological impacts, including enhancing the oxidative damage to cellular components through ROS and chronic inflammation[146]. Zinc deficiency impairs the function of antioxidant enzymes, such as superoxide dismutase (SOD), which depend on zinc as a cofactor. This deficiency exacerbating the pathology of certain diseases, such as cancer and diabetes, due to the decreased antioxidant defense status[146].

Effect of Alcohol Consumption on Maternal Zinc

Chronic alcohol consumption significantly affects zinc metabolism, leading to zinc deficiency, a common metabolic issue among individuals with alcohol use disorder[103]. This deficiency results from decreased zinc intake and absorption, coupled with increased urinary excretion of zinc[103].

Chronic ethanol ingestion impairs net zinc absorption, particularly in the rat ileum, the most active area of zinc uptake as measured by *in vivo* perfusion. Antonson and Vanderhoof (1983) conducted a study on male Sprague-Dawley rats fed a Lieber-Decarli liquid rat diet for one month, with 36% of their total calories provided as ethanol[5]. Pair-fed controls received these calories as carbohydrate. After this period, zinc absorption was examined in duodenal and ileal segments by *in vivo* perfusion. The results showed a 16% reduction in net zinc absorption in the ileum following chronic ethanol feeding. Duodenal absorption of zinc, which was significantly less than in the ileum, remained unaffected by chronic ethanol

ingestion. These findings indicate that chronic ethanol ingestion impairs net zinc absorption, primarily in the ileum, potentially contributing to zinc deficiency observed after chronic ethanol ingestion.

Moreover, zinc deficiency has been postulated to play a role in the development or progression of ALD. A study by Vatsalya et al. (2018) examined 108 very heavy drinking individuals without clinical evidence of ALD[147]. The researchers found that approximately 40% of the subjects had low serum zinc levels. This group with low zinc levels showed a higher frequency of heavy drinking days in the past 90 days and demonstrated stronger associations between their drinking patterns and zinc deficiency. The study also showed that individuals with low zinc levels had higher aspartate aminotransferase to alanine aminotransferase (AST/ALT) ratios, a marker indicative of alcoholic liver disease, as well as lower albumin levels and higher C-reactive protein (CRP) levels, suggesting impaired liver function and increased inflammation.

Interaction between PAE and Zinc and Effect on Fetal Development and Outcomes

This relationship between alcohol intake and zinc has implications for both maternal and fetal health, as well as the potential development of FASD. Research has shown that zinc deficiency is prevalent among pregnant women consuming alcohol. Flynn et al. (1981) reported lower plasma zinc levels in pregnant women who consumed alcohol compared to control pregnant women[148]. They observed an inverse relationship between plasma zinc levels and the expression of FAS, suggesting a potential link between embryonic zinc deficiency and FAS.

When ethanol is consumed, it triggers an acute phase response that results in sequestration of plasma zinc to the maternal liver. This sequestration is a protective mechanism in which MT binds with zinc[3,149]. However, this process results in a reduction in plasma zinc levels. This reduction in plasma zinc leads to decreased availability of zinc for placental transport. The placenta plays a crucial role in supplying the fetus with necessary nutrients,

including zinc. When the availability of zinc for placental transport is decreased, it can result in fetal zinc deficiency and teratogenesis[3,150].

Further evidence supports the acute phase response hypothesis and the role of zinc in ethanol-induced fetal abnormalities. Studies using MT knockout mice have shown that these animals do not exhibit increased fetal dysmorphology when exposed to ethanol during organogenesis, unlike their wild-type counterparts. MT knockout mice maintained normal plasma zinc levels and zinc transfer to the fetus after ethanol exposure[151,152]. Additionally, dietary zinc supplementation throughout pregnancy has been shown to protect against ethanol-mediated developmental damage. In a study by Summers, Rofe, and Coyle (2009), zinc-supplemented mice exposed to ethanol showed a significantly lower incidence of fetal abnormalities compared to those exposed to ethanol alone[150]. Zinc supplementation also improved postnatal survival rates[150]. These findings underscore the protective role of zinc against ethanol-induced fetal dysmorphology and postnatal mortality, although the exact mechanisms—whether MT-dependent or independent—require further investigation.

1.6.6 Copper

Effect of Pregnancy on Maternal Copper

Pregnancy alters copper metabolism to meet increased demands for maternal tissue expansion and fetal growth. Maternal serum copper concentrations can double during gestation, a physiological change driven primarily by an estrogen-stimulated increase in ceruloplasmin. This rise in protein-bound copper does not reflect an excess of bioavailable copper; rather, it is an adaptation to facilitate transport to the developing fetus[153].

To support fetal copper accretion, which is critical for the development of the brain, heart, and skeletal system, the Recommended Dietary Allowance (RDA) for copper increases from 900 µg/day for non-pregnant women to 1000 µg/day during pregnancy. The Tolerable Upper Intake Level (UL) is 10,000 µg/day. Achieving the recommended intake is vital, as inadequate copper status during this critical period can have persistent adverse effects on

the offspring[153].

Effect of Maternal Copper on Fetal Development

Mice lacking specific copper transporters and chaperones, including Ctr1, Cox17, and Atox-1, showed severe malformations and high rates of embryonic and perinatal death[154–157]. The Ctr1 gene encodes a component of the copper transport machinery. Mice without Ctr1 displayed significant growth and developmental defects, dying in utero during mid-gestation[155]. Ctr1-/- embryos were severely underdeveloped and morphologically abnormal, indicating that Ctr1 serves as a primary pathway for copper uptake in mammalian cells[156]. Mice with a disrupted Atox1 locus, which encodes a metallochaperone involved in intracellular copper trafficking, struggled to survive after birth, with 45% dying before weaning. Surviving animals exhibited stunted growth, loose skin, reduced pigmentation, and seizures due to perinatal copper deficiency[154]. Mutations in the ATP7A gene disrupt copper metabolism in infants, causing Menkes disease, an X-linked disorder that impairs copper transport across cell membranes. Affected infants show low copper concentrations in brain and liver tissues, leading to severe brain, vessel, bone, and skin abnormalities, paired with growth failure. Infants with minimal ATP7A activity rarely survive past age three[153].

Copper deficiency affects brain development, causing neuropathological effects across species. Studies on guinea pigs, which undergo substantial in utero myelination, showed high rates of ataxia and brain abnormalities in offspring when mothers consumed copper-deficient diets[158]. Rat offspring from copper-deficient mothers exhibited hyperirritability and convulsive seizures[159]. These findings align with observations in domestic ruminants, where enzootic ataxia, characterized by very low blood copper levels, is common[160,161]. Another study found that copper deficiency in developing rats led to enlarged and abnormally shaped brain mitochondria with reduced cytochrome c oxidase content, impaired oxidative capacity, and altered energy metabolism, suggesting significant impacts on mitochondrial function and morphology[162].

Copper deficiency significantly impacts fetal growth and development, with decreased organ weights observed in copper-deficient offspring. This reduction in organ weight, particularly in the fetal liver, lung, and kidney, is strongly correlated with the decrease in maternal liver copper levels[163]. Copper deficiency may contribute to oxidative stress and reduced antioxidant capacity in the fetus, as evidenced by lower catalase-like activity and reduced SOD levels in copper-deficient fetuses[164]. Importantly, the effects of prenatal copper deficiency can persist even with postnatal supplementation, highlighting the critical role of adequate copper nutrition during pregnancy. For instance, offspring born to copper-deficient dams exhibit long-term neurobehavioral abnormalities, such as a markedly decreased auditory startle response, even when weaned onto a control diet[165]. Clinical studies have shown a negative correlation between maternal serum copper levels and fetal head circumference, as well as between cord blood copper levels and neonatal birth weight, although it remains unclear whether high copper levels are a cause or consequence of poor fetal growth[166].

The influence of copper on vascular development is evidenced in infants with Menkes disease as well as by *in vitro* mouse studies. Embryos cultured in copper-deficient media display yolk sac and vascular anomalies, characterized by persistent blood pools and hemorrhagic vessels, suggesting incomplete and immature vascularization[167,168]. This abnormal vasculature could potentially disrupt the essential nutrient supply during embryogenesis, leading to developmental defects. Copper deficiency results in low SOD activity and increased superoxide anion concentrations, which can interact with nitric oxide (NO), to reduce NO levels and impact NO-dependent vessel relaxation. The addition of NO donors reverses resulting developmental abnormalities[169]. The influence of copper on angiogenesis, the process of new capillary branch formation from existing blood vessels, has been demonstrated through its effects on the expression and biological activity of vascular endothelial growth factor (VEGF) and angiogenin[170]. Advanced imaging techniques, such as X-ray fluorescence microscopy, have shown that during angiogenesis, cellular copper is redistributed from perinuclear areas to the tips of filopodia and across the cell membrane into the extracellular space, suggesting

a direct role for copper in regulating this process[171].

Effect of Maternal Copper on Offspring Outcomes

Research from Peru in the 1960s first characterized the clinical signs of copper deficiency in malnourished infants recovering with milk-based formulas. Similar patterns later emerged in premature infants, those receiving total parenteral nutrition, and infants fed special diets or unmodified cow milk[172].

LBW and very low birth weight (VLBW) preterm infants face high risks of copper deficiency since they fail to undergo the crucial late-gestational copper accumulation in the fetal liver that normally supplies copper during early milk feeding. VLBW infants typically develop signs between 8-10 weeks postnatal, including poor growth, edema, and osteoporosis, while LBW preterm infants show skeletal changes, neutropenia, anemia, and skin hypopigmentation. Research demonstrates that LBW infants with rapid weight gain in their first 6 months exhibited low erythrocyte CuZn superoxide dismutase (CuZnSOD) activity, suggesting both its utility as a copper status indicator and increased copper depletion risks in fast-growing LBW infants - a status that correlated with increased DNA oxidative damage[153].

Copper deficiency disrupts both cardiac function and embryonic development. In adult hearts, copper deficiency causes hypertrophy, and fibrosis[173]. The cardiac pathology stems from reduced CuZnSOD activity, increased oxidative stress, and impaired energy metabolism through decreased cytochrome-c oxidase function[174]. These changes elevate cardiac NO production through increased endothelial and inducible NO synthase expression[173]. MT overexpression in the heart in transgenic mice guards against cardiac oxidative injury from oxidative damage and slows hypertrophy progression[174]. In cardiac ultrastructure, marginal copper deficiency causes mitochondrial abnormalities, increased lipid deposits, and basal laminae alterations[175].

Effect of Alcohol Consumption on Maternal Copper

A case study of a 71-year-old man with a history of heavy alcohol consumption diagnosed with copper deficiency marked the first report linking alcohol abuse to copper deficiency[176]. Research on ALD strengthened this connection through investigations of dietary copper's role in ALD pathogenesis. The research showed that both adequate and supplemental dietary copper reduced alcohol-induced liver and intestine injury, while marginal dietary copper worsened these conditions. The protective mechanisms involved copper's regulation of intestinal hypoxia-inducible factor 1α (HIF-1α) signaling and oxidative stress. Alcohol consumption damaged intestinal villi, reduced HIF-1α expression, and impaired barrier function, effects that copper supplementation mitigated[177]. Studies on rats during gestation and lactation showed that ethanol intake reduced copper concentrations in both dam and pup livers. The research pointed to enhanced biliary copper excretion from ethanol consumption as a potential mechanism for decreased copper status. The data aligned with observations in alcoholics showing changes in ceruloplasmin (CP) activity[6].

Interaction between PAE and Copper and Effect on Fetal Development and Outcomes

Copper, along with nutrients like folate and zinc, is essential for the normal development of the central nervous system (CNS). Deficiencies in these nutrients, frequently seen in individuals with alcohol use disorder, can lead to abnormal CNS development and severe malformations[178,179]. These deficiencies often result from the replacement of food-derived calories with ethanol-derived calories, leading to inadequate intake of essential nutrients[180]. This issue is particularly significant during pregnancy, where heavy alcohol consumption can cause multiple nutrient deficiencies, increasing the risk of FASD. The teratogenic effects of these deficiencies can be exacerbated by their interactions. For example, rat litters from dams fed diets low in both zinc and folate showed a higher frequency of dysmorphogenesis compared to those with diets deficient in only one nutrient[181].

A study by Lee and Cerklewski (1990) on pregnant rats fed a liquid diet with different levels of copper and ethanol demonstrated that maternal ethanol intake reduced copper concentrations in both maternal and pup livers, especially when dietary copper was low[182]. This effect was also observed in the copper content of the pup liver MT fraction, highlighting the liver's role in maintaining copper homeostasis. The study suggested that the combination of low dietary copper, high copper demand during pregnancy and lactation, and ethanol consumption could significantly impact both mothers and offspring.

Copper deficiency during fetal development causes structural and biochemical abnormalities through disrupted free radical defense, connective tissue metabolism, and energy production. Studies link maternal alcohol consumption to reduced fetal copper levels and decreased SOD activity. Research with transgenic mice demonstrates that increased CuZn-SOD expression reduces developmental damage from diabetes, pointing to copper-dependent enzymes' protective role against oxidative stress. Women of reproductive age typically consume less than the recommended 1.5-3.0 mg Cu/d, creating a baseline deficiency risk that alcohol consumption compounds. Laboratory studies document that copper-deficient embryos develop swollen hindbrains, vascular abnormalities, blood pooling, and cardinal vein distention. These findings suggest a synergistic relationship between PAE and copper deficiency in causing developmental abnormalities[179].

1.6.7 Iron

Effect of Pregnancy on Maternal Iron

Pregnancy increases iron demands for fetal and placental development[183]. These requirements vary across trimesters. The cessation of menstruation reduces iron needs in the first trimester to 0.8 mg/day, below pre-pregnancy levels. However, maternal red blood cell mass expansion, coupled with placental and fetal growth, raises iron requirements from 4-5 mg/-day in the second trimester to more than 6 mg/day by the third trimester[184]. Meeting these demands requires increased dietary iron absorption and iron store mobilization. Many

women start pregnancy with insufficient iron stores[185]. Iron deficiency and iron deficiency anemia during pregnancy correlate to maternal mortality, premature birth, LBW, and infant neurodevelopmental impairment[186,187], leading to widespread iron supplementation recommendations. Studies using stable or radioactive iron isotopes show increased nonheme iron absorption as pregnancy progresses[188]. Heme absorption exhibits similar patterns[189].

Research shows decreased maternal hepcidin, a peptide hormone central to iron regulation, concentrations during second and third trimesters in humans[190,191] and the second week of gestation in rats[192]. Lower maternal hepcidin enables increased iron circulation through enhanced dietary absorption and store release. A study of 19 pregnant women demonstrated an inverse correlation between third-trimester serum hepcidin and fetal transfer of dietary nonheme and heme iron[189].

The mechanism behind pregnancy-related maternal hepcidin suppression remains unclear. Plasma hemodilution offers a partial explanation but cannot account for the observed decrease magnitude, given only a 30–50% plasma volume increase[183]. This explanation fails to address the substantial hepatic hepcidin messenger RNA suppression observed in animal studies[192]. While hepcidin reaches its lowest levels in pregnant women with iron deficiency, mothers with sufficient iron stores still show low hepcidin at delivery[193,194], suggesting active pregnancy-related suppression.

Effect of Maternal Iron on Pregnancy Outcomes

Maternal iron deficiency during pregnancy can lead to adverse outcomes for both the mother and the offspring. The World Health Organization recommends routine iron supplementation for pregnant women, particularly in regions where malnutrition is prevalent, to mitigate these risks[195,196]. Maternal anemia has been linked to adverse outcomes such as LBW, preterm birth, and decreased infant iron stores, which can impair cognitive development and growth in children[196].

Iron supplementation reduces the incidence of maternal anemia and iron deficiency as

well as the risk of LBW[197]. Early supplementation improves maternal iron status without increasing the risk of haemoconcentration, however, the benefits may vary depending on pre-pregnancy iron status, with iron-deficient women experiencing most improvements[198]. While some concerns exist about potential adverse effects of iron supplementation in iron-sufficient individuals, the overall evidence supports its use, particularly in low-income countries with high prevalence of iron-deficiency[197]. A study conducted in rural Nepal found that prenatal supplementation with folic acid and iron significantly reduced the risk of obstetric complications, such as postpartum hemorrhage and puerperal sepsis[199].

Effect of Maternal Iron on Offspring Outcomes

Iron deficiency without anemia alters cognitive and behavioral performance in children. Brain regions regulate iron concentrations based on local needs, with specific areas requiring higher iron levels[200]. The developmental timing of iron deficiency creates distinct regional vulnerabilities in the brain[201]. Studies in rats show that early-life iron deficiency reduces dopamine receptor density in parts of the brain by 20-30%. Iron repletion normalizes dopamine receptors in most brain regions, but not all, suggesting permanent alterations from early deficiency[201]. Human infants with iron deficiency show increased central conduction times in auditory processing, indicating potential hypomyelination[202].

The evidence linking maternal iron status to offspring health is inconsistent, with some studies finding no clear relationship between maternal iron status and developmental outcomes[203]. In one study, adequate serum ferritin and iron intake in early pregnancy correlated with better offspring working memory and executive function[204]. However, a meta-analysis of randomized controlled trials found minimal impact of universal antenatal iron supplementation on offspring neurodevelopment. The authors suggested the benefits of supplementation vary with population anemia prevalence, promoting targeted rather than universal supplementation in regions with low anemia rates[205].

Effect of Alcohol Consumption on Maternal Iron

Alcohol consumption exhibits complex interactions with iron metabolism. Research demonstrates a biphasic relationship between alcohol intake and iron status. Light to moderate alcohol consumption, defined as up to two drinks per day, can reduce iron deficiency and iron deficiency anemia risk by 40%[206]. Heavy alcohol intake, exceeding two drinks daily, can disrupt iron homeostasis through multiple mechanisms. Alcohol metabolism suppresses hepatic hepcidin transcription, which increases duodenal iron transporter protein expression, specifically divalent metal transporter 1 (DMT1) and ferroportin, leading to enhanced intestinal iron absorption[207].

Patients with ALD display elevated serum iron indices and hepatic iron concentrations. Iron acts synergistically with alcohol to amplify oxidative stress and lipid peroxidation in hepatic tissue. In Kupffer cells, increased iron content activates nuclear factor-kappa B (NF-κB) and promotes tumor necrosis factor-alpha (TNF-α) expression, contributing to liver injury. Iron chelation therapy mitigates these inflammatory responses, supporting iron's pathogenic role[207]. Antioxidant supplementation with vitamin E or N-acetylcysteine, a precursor to the antioxidant, glutathione, blocks alcohol's effects on hepcidin expression and subsequent iron transport alterations[208].

Interaction between PAE and Maternal Iron Status and Effect on Fetal and Offspring Development and Outcomes

Maternal iron status and PAE interact to influence fetal development and can contribute to FASD. Studies demonstrate that iron deficiency significantly interacts with alcohol exposure to impair postnatal somatic growth, white matter formation, and associative learning beyond the effects of either condition alone[209]. Clinical research shows that infants born to mothers who engaged in binge drinking during pregnancy face 3.6 times higher risk of iron-deficiency anemia at 12 months compared to infants of non-binge drinking mothers[210]. Heavy-drinking mothers often develop iron deficiency during pregnancy, which is particularly concerning as

fetuses rely on maternal iron stores for their development[210].

In animal models, gestational iron supplementation shows specific benefits in alcohol-exposed pregnancies. Research demonstrates that iron supplementation normalizes brain weight reduction in alcohol-exposed male fetuses, though female litter mates do not show the same improvement. Additionally, iron supplementation reverses alcohol-induced fetal anemia and normalizes red blood cell numbers and hematocrit[211].

Socioeconomic factors influence these outcomes. Higher maternal socioeconomic status correlates with decreased FASD severity and frequency, partly because gestational iron deficiency prevalence decreases as income and education rise, reflecting increased iron supplement use and increased diet quality[209].

1.7 Free Radicals, Oxidative Stress, Ethanol, and Pregnancy

1.7.1 Free Radicals and Oxidative Stress

Free radicals exist as molecules or atoms containing an unpaired electron in a valence shell, including superoxide anion and hydroxyl radical[212]. The unpaired electron makes these substances highly reactive, leading to oxidation or reduction of compounds in chain reactions. These reactions pair unpaired electrons with electrons from other compounds, creating new radicals from non-radical molecules[213]. Free radicals perform vital biological functions through phagocytosis and biochemical reactions, including hydroxylation, carboxylation, and peroxidation. Cellular responses to reactive metabolites depend on concentration, exposure time, antioxidant status, and repair systems activation. Cellular responses include proliferation, cell cycle arrest, senescence, apoptosis, or necrosis[213].

Oxidative stress represents an imbalance between ROS and reactive nitrogen species (RNS) production versus antioxidant defense, also known as a cell's redox state[212]. This

imbalance is associated with cardiovascular diseases, neurodegenerative disorders, diabetes, and cancer. The cellular redox state modulates signal pathways, influencing the cell's fate through various pathways. Modest amounts of free radicals support normal physiological functions, making complete suppression undesirable. The distinction between physiological and pathological effects lacks clear definition, exemplifying the complexity of free radical biology[213].

1.7.2 Ethanol Metabolism

The metabolism of ethanol in the human body occurs through oxidative and non-oxidative pathways. The primary oxidative pathway takes place in hepatocyte cytosol through alcohol dehydrogenase (ADH), a zinc metalloenzyme[214]. ADH genes encode multiple isozymes with distinct kinetic properties and substrate specificities, categorized into Class I, II, and III[215]. The ADH oxidation pathway converts ethanol to acetaldehyde, reducing Nicotinamide adenine dinucleotide (oxidized form) (NAD+) to Nicotinamide adenine dinucleotide (reduced form) (NADH) and creating conditions that predispose cells to metabolic injury[216]. Secondary oxidative pathways include cytochrome P450 2E1 (CYP2E1) and catalase. CYP2E1, present in endoplasmic reticulum and mitochondria, metabolizes ethanol at elevated concentrations and is increased with chronic consumption[217]. Catalase, a peroxisomal enzyme, oxidizes ethanol using hydrogen peroxide. This pathway gains significance during fasting and chronic alcohol use[216]. Non-oxidative metabolism of ethanol produces fatty acid ethyl esters (FAEEs) and phosphatidylethanol[218]. These metabolites persist in serum and tissues after ethanol elimination[219]. High concentrations of plasma FAEEs could be an indicator of chronic alcohol abuse and can be used to differentiate from individuals with a history of acute alcohol abuse[220].

1.7.3 Role of ROS in Fetal Development and Alcohol-Induced Damage

Reactive oxygen species regulate cell signaling. These signals direct tissue and organ formation during embryonic development. ROS mediate developmental pathways, such as Wnt signaling, determine cell polarity, fate, and embryo patterning[221]. Hydrogen peroxide acts as a signaling molecule in growth factor pathways[222]. Blood vessel formation requires ROS for endothelial cell differentiation, vessel sprouting, and vascular smooth muscle cell migration[223]. Through modification of cysteine residues in proteins, ROS regulate enzymatic activities[221]. This controlled production of ROS guides normal development through precise spatial and temporal regulation.

The mitochondrial electron transport chain produces ROS in cells, including those in embryonic and fetal development. During oxidative phosphorylation, Complex I and Complex III release superoxide anions. The mitochondrial matrix contains manganese-SOD, which converts superoxide to hydrogen peroxide, and glutathione peroxidase (GPx), which reduces hydrogen peroxide to water. Oxidative stress manifests when ROS production surpasses antioxidant capacity. Low metabolic rates in developing embryos suggest an evolutionary mechanism protecting against ROS-induced cellular damage[224].

The physiological functions of ROS are counterbalanced by their potential for developmental harm, a risk magnified in genetically or environmentally susceptible offspring. When cells produce excess ROS or fail to remove it, oxidative stress damages lipids, proteins, and DNA. This damage can disrupt development, causing birth defects. Genetic predisposition amplifies ROS effects. Mutations affecting antioxidative enzymes impair ROS detoxification, raising oxidative damage risk. Animal studies demonstrate this through genetic ROS detoxification deficiencies. For example, glucose-6-phosphate dehydrogenase (G6PD) and catalase-null mice show heightened sensitivity to ROS-induced birth defects[225,226].

Ethanol metabolism during pregnancy creates a complex cascade of oxidative events that significantly impact fetal development. The metabolic breakdown produces acetaldehyde and

oxygen-derived compounds - superoxide, hydrogen peroxide, and hydroxyl radicals. These products oxidize macromolecules, damage cell membranes, and reduce reduced glutathione (GSH) levels, weakening cellular defenses[4].

NADPH oxidases (NOXs) are a class of enzymes that generate superoxide anions. NOXs expression spans multiple cell types, mediating immune responses and cell signaling pathways. NOXs activity peaks during embryonic development, generating ROS required for developmental progression. NOXs dysregulation and overactivity can lead to excessive ROS production, surpassing the embryo's antioxidative capacity and resulting in oxidative stress. Ethanol triggers NOXs activation and ROS generation[227].

The brain during development exhibits heightened susceptibility to alcohol-induced oxidative stress. This vulnerability stems from its oxygen metabolic rate, which surpasses all other tissues. The brain's composition includes high concentrations of autoxidizable neurotransmitters and unsaturated fatty acids - both serving as ROS substrates. When these components react with ROS, they produce additional reactive species (superoxides, quinones, and semiquinones), triggering oxidative cascades. The brain's reduced levels of antioxidant enzymes compared to other tissues increase its vulnerability to ROS-mediated damage[4].

The placenta metabolizes and eliminates ethanol and acetaldehyde through ADH and aldehyde dehydrogenase (ALDH), reducing concentrations in maternal plasma and fetal exposure, however, elimination is not immediate[227]. The fetus lacks effective alcohol metabolism mechanisms, with enzyme production increasing during development. Fetal CYP2E1 reaches 40% of adult levels in the second trimester and 80% in the third trimester. This means alcohol metabolism in the fetus occurs at half the rate of maternal metabolism[228].

Animal experiments corroborate the pathogenic role of ROS. These studies measured ethanol enhanced ROS formation with fluorescent probes and documented oxidative damage to cellular macromolecules. Research showed protection against this damage and embryopathies through several approaches: vitamin E administration, iron chelator application, upregulation of antioxidative enzymes like SOD, and polyethylene glycol (PEG)-conjugated

catalase treatment. This research advances our understanding of ethanol's teratogenic mechanisms and suggests paths toward reducing PAE impacts[225,227]. Animal studies demonstrated that the application of antioxidants can rescue some phenotypes induced by ethanol toxicity. Nanomaterial research shows potential for antioxidant therapy, though human application requires further investigation[4].

1.8 Public Health Interventions for FASD

FASD prevention campaigns face persistent challenges in reducing PAE. Public health interventions, including warning labels mandated by the Alcohol Beverage Labeling Act of 1988, have increased public awareness but failed to modify drinking patterns among high-risk populations. Research indicates that warning labels stimulate discussions between pregnant women and healthcare providers, yet prove largely ineffective in changing the behavior of heavy drinkers[229]. Recent data shows that nearly 13% of pregnant women continue to consume alcohol during pregnancy, despite increased public knowledge about the relationship between drinking and birth defects[230].

Motivational interviewing (MI) applies across multiple behavioral domains, showing efficacy in both reducing maladaptive behaviors and promoting health changes[231]. In pregnancy intervention studies, MI paired with contraception counseling reduced alcohol-exposed pregnancy risk through dual behavior modification - drinking reduction and contraception use[232]. Single-session MI interventions showed less impact on alcohol-exposed pregnancy risk compared to interventions with multiple sessions[233]. A systematic review of psychosocial interventions identified potential behavior change techniques driving positive outcomes, including action planning, behavioral contracts, and self-monitoring prompts[234]. Recent meta-analyses report brief interventions increase the odds of pregnancy alcohol abstinence rates by 56% and modestly reduce preterm birth risk, though these findings carried low certainty ratings from the authors due to study heterogeneity and methodological limitations[235].

Research examining FASD prevention has evaluated other intervention approaches as well. A systematic review showed brief interventions produced stronger outcomes among specific subgroups: pregnant women with higher baseline alcohol consumption, those who participated with partners, and individuals using multiple substances[236]. For pregnant women using multiple substances, case management programs targeting substance use and social support demonstrated preliminary positive results[236]. The authors identified gaps in current intervention methods: case management and home visits lacked empirical support, while MI studies showed minimal impact due to small sample sizes and low baseline alcohol use. Digital interventions, including text messaging and computer-based screening, showed promise but require additional evaluation[237].

Research into nutritional supplementation for FASD prevention has yielded promising results. A clinical trial in Ukraine demonstrated that choline supplementation combined with prenatal multivitamins improved infant learning mechanisms and memory encoding[42]. A randomized, double-blind study in Cape Town showed that pregnant women receiving 2g daily choline supplementation had infants with enhanced eyeblink conditioning responses and improved visual recognition memory at 12 months compared to the placebo group[85]. These studies point to choline's role in protecting fetal brain development through metabolic pathways, specifically by preventing alcohol-related depletion of dimethylglycine during neurogenesis[42].

In addition to choline, MVM supplementation has shown potential in mitigating the risks associated with PAE and oxidative stress linked to FASD. Coles et al. (2015) found that maternal MVM supplementation during pregnancy was associated with improved cognitive outcomes in infants at 6 months, particularly those exposed to higher levels of alcohol[87]. The study demonstrated that infants whose mothers received MVM supplements exhibited higher Mental Developmental Index (MDI) scores compared to those whose mothers did not receive supplementation, suggesting a protective effect against alcohol-induced neurodevelopmental deficits. Similarly, Avalos et al. (2011) observed that among women who did not take MVM

supplements, low-to-moderate alcohol consumption during early pregnancy was associated with an increased risk of small-for-gestational-age births[238]. In contrast, women who took MVM supplements did not show this increased risk indicating that such supplementation may mitigate some of the adverse effects of PAE on fetal growth[238].

The protective role of MVM supplements may be partly attributed to the reduction of oxidative stress induced by alcohol consumption during pregnancy. Antioxidant vitamins such as Vitamin C and E, as well as micronutrients like folic acid, have been shown to attenuate oxidative damage and improve fetal outcomes in animal models of FASD[239]. Therefore, ensuring adequate maternal intake of essential vitamins and minerals during pregnancy may offer a valuable strategy for reducing the impact of PAE on fetal development.

1.9 Conclusion

Maternal nutrition provides a foundation for healthy fetal development, a complex biological process that PAE can profoundly disrupt. This review delineates the relationships between maternal dietary adequacy, alcohol consumption during gestation, the induction of oxidative stress, and the subsequent risk of FASD. Micronutrients play fundamental roles in neurodevelopment and alcohol use can compromise their bioavailability and action, leading to adverse effects on the developing fetus. An imbalance in redox state, or oxidative stress, emerges as a prominent pathway for alcohol-mediated fetal injury. Current FASD prevention approaches demonstrate varied success. A deeper comprehension of the biochemical disturbances, including maternal nutritional and oxidative stress biomarkers, points toward refined strategies to mitigate risks and support improved developmental trajectories for children experiencing PAE.

Bibliography

[1] Svetlana Popova. "Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis". en. In: *The Lancet Global Health* 5.3 (2017), p. 10. DOI: 10.1016/S2214-109X(17)30021-9.

[2] Shannon Lange et al. "Global prevalence of fetal alcohol spectrum disorder among children and youth: a systematic review and meta-analysis". en. In: *Obstetrical & Gynecological Survey* 73.4 (Apr. 2018), pp. 189–191. DOI: 10.1097/01.ogx.0000532194.88210.00.

[3] Carl L. Keen et al. "The plausibility of maternal nutritional status being a contributing factor to the risk for fetal alcohol spectrum disorders: the potential influence of zinc status as an example". en. In: *BioFactors* 36.2 (Mar. 2010), pp. 125–135. DOI: 10.1002/biof.89.

[4] David González-Flores, Antonia Márquez, and Ilda Casimiro. "Oxidative Effects in Early Stages of Embryo Development Due to Alcohol Consumption". en. In: *International Journal of Molecular Sciences* 25.7 (Jan. 2024), p. 4100. DOI: 10.3390/ijms25074100.

[5] Dean L. Antonson and Jon A. Vanderhoof. "Effect of chronic ethanol ingestion on zinc absorption in rat small intestine". en. In: *Digestive diseases and sciences* 28.7 (July 1983), pp. 604–608. DOI: 10.1007/BF01299920.

[6] J H Baek and F L Cerklewski. "Influence of maternal ethanol ingestion on copper utilization during gestation and lactation in the rat". In: *Fed. Proc., Fed. Am. Soc. Exp. Biol.;* (Mar. 1986).

[7] L. C. Carey et al. "Maternal ethanol exposure is associated with decreased plasma zinc and increased fetal abnormalities in normal but not metallothionein-null mice". en. In: *Alcoholism: Clinical and Experimental Research* 24.2 (Feb. 2000), pp. 213–219.

[8] H. Eugene Hoyme et al. "Updated clinical guidelines for diagnosing fetal alcohol spectrum disorders". en. In: *Pediatrics* 138.2 (Aug. 2016), e20154256. DOI: 10.1542/peds.2015-4256.

[9] Kelly Nash et al. "Improving executive functioning in children with fetal alcohol spectrum disorders". en. In: *Child Neuropsychology* 21.2 (Mar. 2015), pp. 191–209. DOI: 10.1080/09297049.2014.889110.

[10] Debra W. Soh et al. "Self-regulation therapy increases frontal gray matter in children with fetal alcohol spectrum disorder: evaluation by voxel-based morphometry". en. In: *Frontiers in Human Neuroscience* 9 (Mar. 2015). DOI: 10.3389/fnhum.2015.00108.

[11] Julie A. Kable et al. "Improving FASD children's self-regulation: piloting phase 1 of the GoFAR intervention". en. In: *Child & Family Behavior Therapy* 38.2 (Apr. 2016), pp. 124–141. DOI: 10.1080/07317107.2016.1172880.

[12] Claire D. Coles et al. “A metacognitive strategy for reducing disruptive behavior in children with fetal alcohol spectrum disorders: GoFAR pilot”. en. In: *Alcoholism: Clinical and Experimental Research* 39.11 (2015). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/acer.12885, pp. 2224–2233. DOI: 10.1111/acer.12885.

[13] Christie L. M. Petrenko, Mary E. Pandolfino, and Luther K. Robinson. “Findings from the families on track intervention pilot trial for children with fetal alcohol spectrum disorders and their families”. en. In: *Alcoholism: Clinical and Experimental Research* 41.7 (2017), pp. 1340–1351. DOI: 10.1111/acer.13408.

[14] Babatope O. Adebiyi, Ferdinand C. Mukumbang, and Charlene Erasmus. “The distribution of available prevention and management interventions for fetal alcohol spectrum disorder (2007 to 2017): implications for collaborative actions”. en. In: *International Journal of Environmental Research and Public Health* 16.12 (June 2019), p. 2244. DOI: 10.3390/ijerph16122244.

[15] Wendy O. Kalberg et al. “Early-life predictors of fetal alcohol spectrum disorders”. en. In: *Pediatrics* 144.6 (Dec. 2019), e20182141. DOI: 10.1542/peds.2018-2141.

[16] P. May et al. “Characteristic physical traits of first-grade children in the United States with fetal alcohol spectrum disorders (FASD) and associated alcohol and drug exposures”. en. In: *American Journal of Medical Genetics Part A* 188.7 (2022), pp. 2019–2035. DOI: 10.1002/AJMG.A.62738.

[17] Miguel Del Campo et al. “Secondary physical features in children with FASD”. en. In: *European Journal of Medical Genetics* 67 (Feb. 2024), p. 104890. DOI: 10.1016/j.ejmg.2023.104890.

[18] Philip A. May et al. “Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies”. en. In: *Developmental Disabilities Research Reviews* 15.3 (Jan. 2009), pp. 176–192. DOI: 10.1002/ddrr.68.

[19] Ira J. Chasnoff, Anne M. Wells, and Lauren King. “Misdiagnosis and missed diagnoses in foster and adopted children with prenatal alcohol exposure”. en. In: *Pediatrics* 135.2 (Feb. 2015), pp. 264–270. DOI: 10.1542/peds.2014-2171.

[20] Ann P. Streissguth et al. “Risk Factors for Adverse Life Outcomes in Fetal Alcohol Syndrome and Fetal Alcohol Effects”. en-US. In: *Journal of Developmental & Behavioral Pediatrics* 25.4 (Aug. 2004), p. 228.

[21] Philip A. May et al. “The challenges and pitfalls of fetal alcohol spectrum disorders prevalence studies”. en. In: *Alcoholism: Clinical and Experimental Research* 45.12 (2021). tex.litmapsid: 175714355 tex.pubmedid: 34726795, pp. 2468–2470. DOI: 10.1111/ACER.14735.

[22] Philip A. May et al. “Estimating the community prevalence, child traits, and maternal risk factors of fetal alcohol spectrum disorders (FASD) from a random sample of school children.” en. In: *Drug and Alcohol Dependence* 227 (2021). tex.litmapsid: 125570027 tex.pubmedid: 34388579, p. 108918. DOI: 10.1016/J.DRUGALCDEP.2021.108918.

[23] Svetlana Popova et al. “Prevalence of fetal alcohol spectrum disorder among special subpopulations: a systematic review and meta-analysis”. en. In: *Addiction* 114.7 (July 2019), pp. 1150–1172. DOI: 10.1111/add.14598.

[24] Svetlana Popova et al. “Population-based prevalence of fetal alcohol spectrum disorder in Canada”. en. In: *BMC Public Health* 19.1 (Dec. 2019). tex.litmapsid: 107414296 tex.pubmedid: 31253131, p. 845. DOI: 10.1186/s12889-019-7213-3.

[25] Philip A. May et al. “Fetal Alcohol Spectrum Disorders in a Midwestern City: Child Characteristics, Maternal Risk Traits, and Prevalence”. en. In: *Alcoholism: Clinical and Experimental Research* 44.4 (2020). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/acer.14314, pp. 919–938. DOI: 10.1111/acer.14314.

[26] Philip A. May et al. “Fetal Alcohol Spectrum Disorders in a Southeastern County of the United States: Child Characteristics and Maternal Risk Traits”. en. In: *Alcoholism: Clinical and Experimental Research* 44.4 (2020). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/acer.14313, pp. 939–959. DOI: 10.1111/acer.14313.

[27] Philip A. May et al. “Fetal Alcohol Spectrum Disorders in a Rocky Mountain Region City: Child Characteristics, Maternal Risk Traits, and Prevalence”. en. In: *Alcoholism: Clinical and Experimental Research* 44.4 (2020), pp. 900–918. DOI: 10.1111/acer.14315.

[28] Philip A. May et al. “The prevalence of fetal alcohol spectrum disorders in rural communities in South Africa: a third regional sample of child characteristics and maternal risk factors”. en. In: *Alcoholism: Clinical and Experimental Research* 46.10 (Oct. 2022), pp. 1819–1836. DOI: 10.1111/acer.14922.

[29] Francesco Sessa et al. “Understanding the relationship between fetal alcohol spectrum disorder (FASD) and criminal justice: a systematic review”. en. In: *Health Care* 10.1 (Jan. 2022), p. 84. DOI: 10.3390/healthcare10010084.

[30] Jacob R. Greenmyer et al. “A multicountry updated assessment of the economic impact of fetal alcohol spectrum disorder: costs for children and adults”. en. In: *Journal of Addiction Medicine* 12.6 (Nov. 2018), pp. 466–473. DOI: 10.1097/ADM.0000000000000438.

[31] Mir M. Ali, Larry Burd, and Kristina D. West. *Estimating costs of health care for children with fetal alcohol spectrum disorder in a large health insurance claims database*. en. Aug. 2022. DOI: 10.21203/rs.3.rs-1930471/v1.

[32] Katherine Flannigan et al. “Responding to the unique complexities of fetal alcohol spectrum disorder”. en. In: *Frontiers in Psychology* 12 (Jan. 2022), p. 778471. DOI: 10.3389/fpsyg.2021.778471.

[33] K. Abu-Saad and D. Fraser. “Maternal nutrition and birth outcomes”. en. In: *Epidemiologic Reviews* 32.1 (Apr. 2010), pp. 5–25. DOI: 10.1093/epirev/mxq001.

[34] Zohra S. Lassi et al. “PROTOCOL: effects of nutritional interventions during pregnancy on birth, child health, and development outcomes: a systematic review of evidence from low- and middle-income countries”. en. In: *Campbell Systematic Reviews* 15.1-2 (June 2019), e1019. DOI: 10.1002/cl2.1019.

[35] Xuyang Chen et al. “Maternal dietary patterns and pregnancy outcome”. en. In: *Nutrients* 8.6 (2016). 00000, p. 351. DOI: 10.3390/nu8060351.

[36] Aamer Imdad and Zulfiqar A. Bhutta. “Maternal nutrition and birth outcomes: effect of balanced protein-energy supplementation”. en. In: *Paediatric and Perinatal Epidemiology* 26.s1 (July 2012), pp. 178–190. DOI: 10.1111/j.1365-3016.2012.01308.x.

[37] Usha Ramakrishnan et al. “Effect of Women’s Nutrition before and during Early Pregnancy on Maternal and Infant Outcomes: A Systematic Review”. en. In: *Paediatric and Perinatal Epidemiology* 26.s1 (2012). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1365-3016.2012.01281.x, pp. 285–301. DOI: 10.1111/j.1365-3016.2012.01281.x.

[38] Hunter W. Korsmo, Xinyin Jiang, and Marie A. Caudill. “Choline: exploring the growing science on its benefits for moms and babies”. en. In: *Nutrients* 11.8 (Aug. 2019), p. 1823. DOI: 10.3390/nu11081823.

[39] S. K. Spoelstra et al. “Maternal choline supplementation during pregnancy to promote mental health in offspring”. en. In: *Early Intervention in Psychiatry* 17.7 (July 2023), pp. 643–651. DOI: 10.1111/eip.13426.

[40] Jessica A. Ash et al. “Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice”. en. In: *Neurobiology of Disease* 70 (Oct. 2014), pp. 32–42. DOI: 10.1016/j.nbd.2014.06.001.

[41] Kalynn M. Schulz et al. “Dietary choline supplementation to dams during pregnancy and lactation mitigates the effects of in utero stress exposure on adult anxiety-related behaviors”. en. In: *Behavioural Brain Research* 268 (July 2014), pp. 104–110. DOI: 10.1016/j.bbr.2014.03.031.

[42] J.A. Kable et al. “The impact of micronutrient supplementation in alcohol-exposed pregnancies on information processing skills in Ukrainian infants”. en. In: *Alcohol* 49.7 (Nov. 2015), pp. 647–656. DOI: 10.1016/j.alcohol.2015.08.005.

[43] Kosuke Kawai et al. “Maternal multiple micronutrient supplementation and pregnancy outcomes in developing countries: meta-analysis and meta-regression”. en. In: *Bulletin of the World Health Organization* 89.6 (June 2011), 402–411B. DOI: 10.2471/BLT.10.083758.

[44] Megan W. Bourassa et al. “Review of the evidence regarding the use of antenatal multiple micronutrient supplementation in low- and middle-income countries”. en. In: *Annals of the New York Academy of Sciences* 1444.1 (May 2019), pp. 6–21. DOI: 10.1111/nyas.14121.

[45] Christina Oh, Emily Keats, and Zulfiqar Bhutta. “Vitamin and mineral supplementation during pregnancy on maternal, birth, child health and development outcomes in low- and middle-income countries: a systematic review and meta-analysis”. en. In: *Nutrients* 12.2 (Feb. 2020), p. 491. DOI: 10.3390/nu12020491.

[46] Parul Christian. "Micronutrients, birth weight, and survival". en. In: *Annual Review of Nutrition* 30.1 (July 2010), pp. 83–104. DOI: 10.1146/annurev.nutr.012809.104813.

[47] Delan Devakumar et al. "Maternal antenatal multiple micronutrient supplementation for long-term health benefits in children: a systematic review and meta-analysis". en. In: *BMC Medicine* 14.1 (Dec. 2016), p. 90. DOI: 10.1186/s12916-016-0633-3.

[48] Nathalie Irvine et al. "Prenatal Folate and Choline Levels and Brain and Cognitive Development in Children: A Critical Narrative Review". en. In: *Nutrients* 14.2 (Jan. 2022), p. 364. DOI: 10.3390/nu14020364.

[49] Corneliu N. Craciunescu, Amy R. Johnson, and Steven H. Zeisel. "Dietary Choline Reverses Some, but Not All, Effects of Folate Deficiency on Neurogenesis and Apoptosis in Fetal Mouse Brain". en. In: *The Journal of Nutrition* 140.6 (June 2010), pp. 1162–1166. DOI: 10.3945/jn.110.122044.

[50] N.M. Jadavji et al. "MTHFR deficiency or reduced intake of folate or choline in pregnant mice results in impaired short-term memory and increased apoptosis in the hippocampus of wild-type offspring". en. In: *Neuroscience* 300 (Aug. 2015), pp. 1–9. DOI: 10.1016/j.neuroscience.2015.04.067.

[51] Rola Hammoud et al. "Choline and Folic Acid in Diets Consumed during Pregnancy Interact to Program Food Intake and Metabolic Regulation of Male Wistar Rat Offspring". en. In: *The Journal of Nutrition* 151.4 (Apr. 2021), pp. 857–865. DOI: 10.1093/jn/nxaa419.

[52] Sherry A. Ferguson et al. "Behavioral effects of prenatal folate deficiency in mice". en. In: *Birth Defects Research Part A: Clinical and Molecular Teratology* 73.4 (Apr. 2005), pp. 249–252. DOI: 10.1002/bdra.20111.

[53] Helene McNulty et al. "Effect of continued folic acid supplementation beyond the first trimester of pregnancy on cognitive performance in the child: a follow-up study from a randomized controlled trial (FASSTT Offspring Trial)". en. In: *BMC Medicine* 17.1 (Dec. 2019), p. 196. DOI: 10.1186/s12916-019-1432-4.

[54] Hanxiao Chen et al. "Neurodevelopmental effects of maternal folic acid supplementation: a systematic review and meta-analysis". en. In: *Critical Reviews in Food Science and Nutrition* 63.19 (July 2023), pp. 3771–3787. DOI: 10.1080/10408398.2021.1993781.

[55] Leda Chatzi et al. "Effect of high doses of folic acid supplementation in early pregnancy on child neurodevelopment at 18 months of age: the mother–child cohort 'Rhea' study in Crete, Greece". en. In: *Public Health Nutrition* 15.9 (Sept. 2012), pp. 1728–1736. DOI: 10.1017/S1368980012000067.

[56] Jan Krzysztof Blusztajn, Barbara E. Slack, and Tiffany J. Mellott. "Neuroprotective Actions of Dietary Choline". eng. In: *Nutrients* 9.8 (July 2017). 00074. DOI: 10.3390/nu9080815.

[57] Warren H. Meck, Rebecca A. Smith, and Christinal L. Williams. "Pre- and postnatal choline supplementation produces long-term facilitation of spatial memory". en. In: *Developmental Psychobiology* 21.4 (May 1988), pp. 339–353. DOI: 10.1002/dev.420210405.

[58] Melissa J. Glenn et al. "Age-related declines in exploratory behavior and markers of hippocampal plasticity are attenuated by prenatal choline supplementation in rats". en. In: *Brain Research* 1237 (Oct. 2008), pp. 110–123. DOI: 10.1016/j.brainres.2008.08.049.

[59] Warren H Meck and Christina L Williams. "Choline supplementation during prenatal development reduces proactive interference in spatial memory". en. In: *Developmental Brain Research* 118.1-2 (Dec. 1999), pp. 51–59. DOI: 10.1016/S0165-3806(99)00105-4.

[60] Marie A. Caudill et al. "Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: a randomized, double-blind, controlled feeding study". en. In: *The FASEB Journal* 32.4 (Apr. 2018), pp. 2172–2180. DOI: 10.1096/fj.201700692RR.

[61] C. E. Boeke et al. "Choline Intake During Pregnancy and Child Cognition at Age 7 Years". en. In: *American Journal of Epidemiology* 177.12 (June 2013), pp. 1338–1347. DOI: 10.1093/aje/kws395.

[62] Brian T. F. Wu et al. "Early Second Trimester Maternal Plasma Choline and Betaine Are Related to Measures of Early Cognitive Development in Term Infants". en. In: *PLoS ONE* 7.8 (Aug. 2012). Ed. by Pal Bela Szecsi, e43448. DOI: 10.1371/journal.pone.0043448.

[63] Caroline Signore et al. "Choline concentrations in human maternal and cord blood and intelligence at 5 y of age". en. In: *The American Journal of Clinical Nutrition* 87.4 (Apr. 2008), pp. 896–902. DOI: 10.1093/ajcn/87.4.896.

[64] Valentina Medici and Charles H. Halsted. "Folate, alcohol, and liver disease". en. In: *Molecular Nutrition & Food Research* 57.4 (Apr. 2013), pp. 596–606. DOI: 10.1002/mnfr.201200077.

[65] Bogdan Cylwik and Lech Chrostek. "Interactions Between Alcohol and Folate". en. In: *Molecular Aspects of Alcohol and Nutrition*. Elsevier, 2016, pp. 157–169. DOI: 10.1016/B978-0-12-800773-0.00013-6.

[66] J. Villanueva et al. "Membrane and tissue distribution of folate binding protein in pig". eng. In: *The American Journal of Physiology* 275.5 (Nov. 1998), R1503–1510. DOI: 10.1152/ajpregu.1998.275.5.R1503.

[67] J. A. Villanueva, A. M. Devlin, and C. H. Halsted. "Reduced folate carrier: tissue distribution and effects of chronic ethanol intake in the micropig". eng. In: *Alcoholism, Clinical and Experimental Research* 25.3 (Mar. 2001), pp. 415–420.

[68] Nissar Ahmad Wani and Jyotdeep Kaur. "Reduced levels of folate transporters (PCFT and RFC) in membrane lipid rafts result in colonic folate malabsorption in chronic alcoholism". eng. In: *Journal of Cellular Physiology* 226.3 (Mar. 2011), pp. 579–587. DOI: 10.1002/jcp.22525.

[69] Edward R. Eichner and Robert S. Hillman. "Effect of Alcohol on Serum Folate Level". en. In: *Journal of Clinical Investigation* 52.3 (Mar. 1973), pp. 584–591. DOI: 10.1172/JCI107219.

[70] T. Tamura et al. "Hepatic folate metabolism in the chronic alcoholic monkey". eng. In: *The Journal of Laboratory and Clinical Medicine* 97.5 (May 1981), pp. 654–661.

[71] Kenneth E. McMartin et al. "Effects of Chronic Ethanol And Diet Treatment on Urinary Folate Excretion and Development of Folate Deficiency in the Rat". en. In: *The Journal of Nutrition* 119.10 (Oct. 1989), pp. 1490–1497. DOI: 10.1093/jn/119.10.1490.

[72] Abid Hamid and Jyotdeep Kaur. "Decreased Expression of Transporters Reduces Folate Uptake across Renal Absorptive Surfaces in Experimental Alcoholism". en. In: *Journal of Membrane Biology* 220.1-3 (Dec. 2007), pp. 69–77. DOI: 10.1007/s00232-007-9075-3.

[73] Sarah E. Steane, James S. M. Cuffe, and Karen M. Moritz. "The role of maternal choline, folate and one-carbon metabolism in mediating the impact of prenatal alcohol exposure on placental and fetal development". en. In: *The Journal of Physiology* 601.6 (Mar. 2023), pp. 1061–1075. DOI: 10.1113/JP283556.

[74] Gerald Klatskin, Willard A. Krehl, and Harold O. Conn. "The Effect of Alcohol On The Choline Requirement". en. In: *Journal of Experimental Medicine* 100.6 (Dec. 1954), pp. 605–614. DOI: 10.1084/jem.100.6.605.

[75] S. F. Daft, W. H. Sebrell, and R. D. Lillie. "Prevention by Cystine or Methionine of Hemorrhage and Necrosis of the Liver in Rats". en. In: *Experimental Biology and Medicine* 50.1 (May 1942), pp. 1–5. DOI: 10.3181/00379727-50-13682.

[76] C. H. Best et al. "Liver Damage Produced by Feeding Alcohol or Sugar and its Prevention by Choline". en. In: *BMJ* 2.4635 (Nov. 1949), pp. 1001–1006. DOI: 10.1136/bmj.2.4635.1001.

[77] John A. Thompson and Ronald C. Reitz. "Studies On The Acute and Chronic Effects of Ethanol Ingestion on Choline Oxidation". en. In: *Annals of the New York Academy of Sciences* 273.1 Work in Progr (May 1976), pp. 194–204. DOI: 10.1111/j.1749-6632.1976.tb52882.x.

[78] S. C. Allen. "The effect of longterm alcohol exposure on liver choline dehydrogenase activity". eng. In: *Medical Journal of Zambia* 15.2 (1981), pp. 34–37.

[79] Laura K. Cole, Jean E. Vance, and Dennis E. Vance. "Phosphatidylcholine biosynthesis and lipoprotein metabolism". en. In: *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1821.5 (May 2012), pp. 754–761. DOI: 10.1016/j.bbalip.2011.09.009.

[80] Ibrahim Sogut et al. “Prenatal alcohol–induced neuroapoptosis in rat brain cerebral cortex: protective effect of folic acid and betaine”. en. In: *Child's Nervous System* 33.3 (Mar. 2017), pp. 407–417. DOI: 10.1007/s00381-016-3309-6.

[81] Uttam Shrestha and Mandavi Singh. “Effect of folic acid in prenatal alcohol induced behavioral impairments in Swiss albino mice”. en. In: *Annals of Neurosciences* 20.4 (Oct. 2013). DOI: 10.5214/ans.0972.7531.200403.

[82] Nirelia M. Idrus, Kristen R. Breit, and Jennifer D. Thomas. “Dietary choline levels modify the effects of prenatal alcohol exposure in rats”. eng. In: *Neurotoxicology and Teratology* 59 (Feb. 2017). 00023, pp. 43–52. DOI: 10.1016/j.ntt.2016.11.007.

[83] Jennifer D. Thomas et al. “Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats”. en. In: *Birth Defects Research Part A: Clinical and Molecular Teratology* 88.10 (Oct. 2010), pp. 827–837. DOI: 10.1002/bdra.20713.

[84] Riley T. Bottom, Charles W. Abbott, and Kelly J. Huffman. “Rescue of ethanol-induced FASD-like phenotypes via prenatal co-administration of choline”. en. In: *Neuropharmacology* 168 (May 2020), p. 107990. DOI: 10.1016/j.neuropharm.2020.107990.

[85] Sandra W. Jacobson et al. “Efficacy of Maternal Choline Supplementation During Pregnancy in Mitigating Adverse Effects of Prenatal Alcohol Exposure on Growth and Cognitive Function: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial”. en. In: *Alcoholism: Clinical and Experimental Research* 42.7 (July 2018). 00044, pp. 1327–1341. DOI: 10.1111/acer.13769.

[86] Fleur L. Warton et al. “Maternal choline supplementation mitigates alcohol exposure effects on neonatal brain volumes”. en. In: *Alcoholism: Clinical and Experimental Research* 45.9 (Sept. 2021), pp. 1762–1774. DOI: 10.1111/acer.14672.

[87] Claire D. Coles et al. “Dose and Timing of Prenatal Alcohol Exposure and Maternal Nutritional Supplements: Developmental Effects on 6-Month-Old Infants”. en. In: *Maternal and Child Health Journal* 19.12 (Dec. 2015), pp. 2605–2614. DOI: 10.1007/s10995-015-1779-x.

[88] Lindsay C. Czuba et al. “Plasma retinoid concentrations are altered in pregnant women”. en. In: *Nutrients* 14.7 (Mar. 2022), p. 1365. DOI: 10.3390/nu14071365.

[89] Michael J. Dibley and David A. Jeacocke. “Vitamin A in Pregnancy: Impact on Maternal and Neonatal Health”. en. In: *Food and Nutrition Bulletin* 22.3 (Jan. 2001), pp. 267–284. DOI: 10.1177/156482650102200305.

[90] K. P West et al. “Double blind, cluster randomised trial of low dose supplementation with vitamin A or beta carotene on mortality related to pregnancy in Nepal”. en. In: *BMJ* 318.7183 (Feb. 1999), pp. 570–575. DOI: 10.1136/bmj.318.7183.570.

[91] Fred Hale. “The Relation of Vitamin a to Anophthalmos in Pigs”. en. In: *American Journal of Ophthalmology* 18.12 (Dec. 1935), pp. 1087–1093. DOI: 10.1016/S0002-9394(35)90563-3.

[92] James G. Wilson, Carolyn B. Roth, and Josef Warkany. "An analysis of the syndrome of malformations induced by maternal vitamin a deficiency. Effects of restoration of vitamin a at various times during gestation". en. In: *American Journal of Anatomy* 92.2 (Mar. 1953), pp. 189–217. DOI: 10.1002/aja.1000920202.

[93] Margaret Clagett-Dame and Danielle Knutson. "Vitamin A in Reproduction and Development". en. In: *Nutrients* 3.4 (Mar. 2011), pp. 385–428. DOI: 10.3390/nu3040385.

[94] Sharon A. Ross et al. "Retinoids in Embryonal Development". en. In: *Physiological Reviews* 80.3 (July 2000), pp. 1021–1054. DOI: 10.1152/physrev.2000.80.3.1021.

[95] Thomas H. Rosenquist et al. "Nutrient Effects upon Embryogenesis: Folate, Vitamin A and Iodine". en. In: *Nestl Nutrition Workshop Series: Pediatric Program*. Ed. by G. Hornstra, R. Uauy, and X. Yang. Basel: KARGER, 2005, pp. 29–47. DOI: 10.1159/000082592.

[96] S Cocco et al. "Vitamin A deficiency produces spatial learning and memory impairment in rats". en. In: *Neuroscience* 115.2 (Dec. 2002), pp. 475–482. DOI: 10.1016/S0306-4522(02)00423-2.

[97] Susan D. Emmett and Keith P. West. "Gestational vitamin A deficiency: A novel cause of sensorineural hearing loss in the developing world?" en. In: *Medical Hypotheses* 82.1 (Jan. 2014), pp. 6–10. DOI: 10.1016/j.mehy.2013.09.028.

[98] Xuan Zhang et al. "Perinatal Vitamin A Status in Relation to Neurodevelopmental Outcome at two Years of Age". en. In: *International Journal for Vitamin and Nutrition Research* 79.4 (July 2009), pp. 238–249. DOI: 10.1024/0300-9831.79.4.238.

[99] Gillian J Buckley et al. "Cognitive and motor skills in school-aged children following maternal vitamin A supplementation during pregnancy in rural Nepal: a follow-up of a placebo-controlled, randomised cohort". en. In: *BMJ Open* 3.5 (2013), e002000. DOI: 10.1136/bmjopen-2012-002000.

[100] Hasmot Ali et al. "Effect of maternal antenatal and newborn supplementation with vitamin A on cognitive development of school-aged children in rural Bangladesh: a follow-up of a placebo-controlled, randomized trial". en. In: *The American Journal of Clinical Nutrition* 106.1 (July 2017), pp. 77–87. DOI: 10.3945/ajcn.116.134478.

[101] Robin D. Clugston and William S. Blaner. "The Adverse Effects of Alcohol on Vitamin A Metabolism". en. In: *Nutrients* 4.5 (May 2012), pp. 356–371. DOI: 10.3390/nu4050356.

[102] Maria Anna Leo and Charles S. Lieber. "Hepatic Vitamin A Depletion in Alcoholic Liver Injury". en. In: *New England Journal of Medicine* 307.10 (Sept. 1982), pp. 597–601. DOI: 10.1056/NEJM198209023071006.

[103] Charles S Lieber. "ALCOHOL: Its Metabolism and Interaction With Nutrients". en. In: *Annu Rev Nutr* (2000), 20:395–430. DOI: 10.1146/annurev.nutr.20.1.395.

[104] Mayumi Sato and Charles S. Lieber. "Hepatic Vitamin a Depletion after Chronic Ethanol Consumption in Baboons and Rats". en. In: *The Journal of Nutrition* 111.11 (Nov. 1981), pp. 2015–2023. DOI: 10.1093/jn/111.11.2015.

[105] Mary A Grummer and Richard D Zachman. "The Effect of Maternal Ethanol Ingestion on Fetal Vitamin A in the Rat". In: *Pediatric Research* 28.3 (Sept. 1990), pp. 186–189. DOI: 10.1203/00006450-199009000-00002.

[106] Mitchell H Dejonge and Richard D Zachman. "The Effect of Maternal Ethanol Ingestion on Fetal Rat Heart Vitamin A: A Model for Fetal Alcohol Syndrome". In: *Pediatric Research* 37.4 (Apr. 1995), pp. 418–423. DOI: 10.1203/00006450-199504000-00006.

[107] Helly R. Goez, Ori Scott, and Simona Hasal. "Fetal Exposure to Alcohol, Developmental Brain Anomaly, and Vitamin A Deficiency: A Case Report". en. In: *Journal of Child Neurology* 26.2 (Feb. 2011), pp. 231–234. DOI: 10.1177/0883073810380458.

[108] Waleed O. Twal and Maija H. Zile. "Retinoic Acid Reverses Ethanol-Induced Cardiovascular Abnormalities in Quail Embryos". en. In: *Alcoholism: Clinical and Experimental Research* 21.6 (Sept. 1997), pp. 1137–1143. DOI: 10.1111/j.1530-0277.1997.tb04264.x.

[109] Cathy Debier. "Vitamin E During Pre- and Postnatal Periods". en. In: *Vitamins & Hormones*. Vol. 76. Elsevier, 2007, pp. 357–373. DOI: 10.1016/S0083-6729(07)76013-2.

[110] Theresa O Scholl et al. "Vitamin E: maternal concentrations are associated with fetal growth2". en. In: *American Journal of Clinical Nutrition* 84.6 (Dec. 2006), pp. 1442–1448. DOI: 10.1093/ajcn/84.6.1442.

[111] Maret G. Traber. "Vitamin E: necessary nutrient for neural development and cognitive function". en. In: *Proceedings of the Nutrition Society* 80.3 (Aug. 2021), pp. 319–326. DOI: 10.1017/S0029665121000914.

[112] C. Debier and Y. Larondelle. "Vitamins a and E: metabolism, roles and transfer to offspring". en. In: *British Journal of Nutrition* 93.2 (Feb. 2005), pp. 153–174. DOI: 10.1079/BJN20041308.

[113] Galen W. Miller et al. "The -Tocopherol Transfer Protein Is Essential for Vertebrate Embryogenesis". en. In: *PLoS ONE* 7.10 (Oct. 2012). Ed. by Harold A. Burgess, e47402. DOI: 10.1371/journal.pone.0047402.

[114] G. A. Bjørneboe et al. "Effect of heavy alcohol consumption on serum concentrations of fat-soluble vitamins and selenium". eng. In: *Alcohol and Alcoholism (Oxford, Oxfordshire). Supplement* 1 (1987), pp. 533–537.

[115] Emilio González-Reimers et al. "Antioxidant Vitamins and Brain Dysfunction in Alcoholics". en. In: *Alcohol and Alcoholism* 49.1 (Jan. 2014), pp. 45–50. DOI: 10.1093/alcalc/agt150.

[116] Nick Hidiroglou and Rene Madere. "Effect of chronic ethanol consumption upon vitamine E and C tissue status in the guinea pig". en. In: *Nutrition Research* 20.6 (June 2000), pp. 815–827. DOI: 10.1016/S0271-5317(00)00172-X.

[117] Parri Wentzel, Ulf Rydberg, and Ulf J. Eriksson. "Antioxidative Treatment Diminishes Ethanol-Induced Congenital Malformations in the Rat". en. In: *Alcoholism: Clinical and Experimental Research* 30.10 (Oct. 2006), pp. 1752–1760. DOI: 10.1111/j.1530-0277.2006.00208.x.

[118] Alireza Shirpoor et al. "The protective effect of vitamin E against prenatal and early postnatal ethanol treatment-induced heart abnormality in rats: A 3-month follow-up study". en. In: *International Immunopharmacology* 26.1 (May 2015), pp. 72–79. DOI: 10.1016/j.intimp.2015.03.008.

[119] María Sánchez-Campillo et al. "Serum Vitamins A and E at Mid-Pregnancy and Their Relationships with Both Maternal and Cord Blood Antioxidant Status and Perinatal Conditions: The NELA Cohort". en. In: *Annals of Nutrition and Metabolism* 79.3 (2023), pp. 313–325. DOI: 10.1159/000531239.

[120] Shu Qin Wei. "Vitamin D and pregnancy outcomes". en. In: *Current Opinion in Obstetrics & Gynecology* 26.6 (Dec. 2014), pp. 438–447. DOI: 10.1097/gco.0000000000000117.

[121] E. Curtis et al. "Maternal vitamin D supplementation during pregnancy". en. In: *British Medical Bulletin* (2018).

[122] B. Hollis and C. Wagner. "Vitamin D supplementation during pregnancy: improvements in birth outc omes and complications through direct genomic alteration". en. In: *Molecular and Cellular Endocrinology* (2017).

[123] Anne-Louise Ponsonby et al. "Vitamin D status during pregnancy and aspects of offspring health". en. In: *Nutrients* 2.3 (Mar. 2010), pp. 389–407. DOI: 10.3390/nu2030389.

[124] Daphna K. Dror. "Vitamin D status during pregnancy". en. In: *Current Opinion in Obstetrics & Gynecology* 23.6 (Dec. 2011), pp. 422–426. DOI: 10.1097/gco.0b013e32834cb791.

[125] Prue H. Hart et al. "Vitamin D in fetal development: findings from a birth cohort study". en. In: *Pediatrics* 135.1 (Jan. 2015), e167–e173. DOI: 10.1542/peds.2014-1860.

[126] Evelien R. Leffelaar, Tanja G. M. Vrijkotte, and Manon van Eijsden. "Maternal early pregnancy vitamin D status in relation to fetal and neo natal growth: results of the multi-ethnic amsterdam born children and their development cohort". en. In: *British Journal of Nutrition* 104.1 (Mar. 2010), pp. 108–117. DOI: 10.1017/s000711451000022x.

[127] Jazmin E. Hawes et al. "Maternal vitamin D deficiency alters fetal brain development in the BA LB/c mouse". en. In: *Behavioural Brain Research* 286 (June 2015), pp. 192–200. DOI: 10.1016/j.bbr.2015.03.008.

[128] Subina Upadhyaya et al. "Maternal Vitamin D Levels during Pregnancy and Offspring Psychiatric O utcomes: A Systematic Review". en. In: *International Journal of Molecular Sciences* 24.1 (Dec. 2022), p. 63. DOI: 10.3390/ijms24010063.

[129] K. Shankar et al. “Chronic ethanol consumption leads to disruption of vitamin D3 homeosta sis associated with induction of renal 1,25 dihydroxyvitamin D3-24-hyd roxylase (CYP24A1).” en. In: *Endocrinology* (2008).

[130] Janice García-Quiroz et al. “Chronic moderate ethanol intake differentially regulates vitamin D hydroxylases gene expression in kidneys and xenografted breast cancer cells in female mice”. en. In: *Journal of Steroid Biochemistry and Molecular Biology*. 19th Vitamin D Workshop 173 (Oct. 2017), pp. 148–156. DOI: 10.1016/j.jsbmb.2016.09.011.

[131] Russell T. Turner et al. “Chronic alcohol treatment results in disturbed vitamin D metabolism an d skeletal abnormalities in rats.” en. In: *Alcoholism: Clinical and Experimental Research* (1988).

[132] Charles R. Carlson et al. “Vitamin D Deficiency in Pregnant Ukrainian Women: Effects of Alcohol Consumption on Vitamin D Status”. en. In: *Journal of the American College of Nutrition* 36.1 (Jan. 2017), pp. 44–56. DOI: 10.1080/07315724.2016.1174091.

[133] Janet C King. “Determinants of maternal zinc status during pregnancy”. en. In: *The American Journal of Clinical Nutrition* 71.5 (May 2000), 1334S–1343S. DOI: 10.1093/ajcn/71.5.1334s.

[134] C A Swanson and J C King. “Zinc and pregnancy outcome”. en. In: *The American Journal of Clinical Nutrition* 46.5 (Nov. 1987), pp. 763–771. DOI: 10.1093/ajcn/46.5.763.

[135] N. T. Davies and R. B. Williams. “The effect of pregnancy and lactation on the absorption of zinc and lysine by the rat duodenum in situ”. en. In: *British Journal of Nutrition* 38.3 (Nov. 1977), pp. 417–423. DOI: 10.1079/BJN19770106.

[136] Janet Y. Uriu-Adams and Carl L. Keen. “Zinc and reproduction: effects of zinc deficiency on prenatal and early postnatal development”. en. In: *Birth Defects Research Part B-developmental and Reproductive Toxicology* 89.4 (Aug. 2010), pp. 313–325. DOI: 10.1002/bdrb.20264.

[137] W. R. Todd, C. A. Elvehjem, and E. B. Hart. “Zinc in the nutrition of the rat”. In: *American Journal of Physiology-Legacy Content* 107.1 (Dec. 1933). Publisher: American Physiological Society, pp. 146–156. DOI: 10.1152/ajplegacy.1933.107.1.146.

[138] L. S. Hurley and H. Swenerton. “Congenital malformations resulting from zinc deficiency in rats”. en. In: *Experimental Biology and Medicine* 123.3 (Dec. 1966), pp. 692–696. DOI: 10.3181/00379727-123-31578.

[139] Sonja Y. Hess. “National risk of zinc deficiency as estimated by national surveys”. en. In: *Food and Nutrition Bulletin* 38.1 (Mar. 2017). Publisher: SAGE Publications Inc, pp. 3–17. DOI: 10.1177/0379572116689000.

[140] Xiuzhen Liu, Ana M. Adamo, and Patricia I. Oteiza. "Marginal zinc deficiency during gestation and lactation in rats affects oligodendrogenesis, motor performance, and behavior in the offspring". en. In: *Journal of Nutrition* 153.10 (Oct. 2023), pp. 2778–2796. DOI: 10.1016/j.tjnut.2023.08.029.

[141] Rebecca L. Wilson et al. "Association between maternal zinc status, dietary zinc intake and pregnancy complications: a systematic review." en. In: *Nutrients* (2016). tex.litmapsid: 160194546 tex.pubmedid: 27754451. DOI: 10/grzft7.

[142] Sehar Iqbal and Inayat Ali. "Effect of maternal zinc supplementation or zinc status on pregnancy complications and perinatal outcomes: an umbrella review of meta-analyses". en. In: *Heliyon* 7.7 (July 2021), e07540. DOI: 10.1016/j.heliyon.2021.e07540.

[143] Benjamin W. Chaffee and Janet C. King. "Effect of zinc supplementation on pregnancy and infant outcomes: a systematic review". en. In: *Paediatric and Perinatal Epidemiology* 26.s1 (July 2012), pp. 118–137. DOI: 10.1111/j.1365-3016.2012.01289.x.

[144] Enju Liu et al. "Effect of zinc supplementation on growth outcomes in children under 5 years of age". en. In: *Nutrients* 10.3 (Mar. 2018), p. 377. DOI: 10.3390/nu10030377.

[145] Samia A. Nossier et al. "The effect of zinc supplementation on pregnancy outcomes: a double-blind, randomised controlled trial, egypt". en. In: *British Journal of Nutrition* 114.2 (July 2015), pp. 274–285. DOI: 10.1017/S000711451500166X.

[146] Azhar Hussain et al. "Mechanistic impact of zinc deficiency in human development". en. In: *Frontiers in Nutrition* 9 (Mar. 2022), p. 717064. DOI: 10.3389/fnut.2022.717064.

[147] Vatsalya Vatsalya et al. "Association of serum zinc with markers of liver injury in very heavy drinking alcohol-dependent patients". en. In: *The Journal of Nutritional Biochemistry* 59 (Sept. 2018), pp. 49–55. DOI: 10.1016/j.jnutbio.2018.05.003.

[148] Arthur Flynn et al. "Zinc status of pregnant alcoholic women: a determinant of fetal outcome". en. In: *Lancet*. Originally published as Volume 1, Issue 8220 317.8220 (Mar. 1981), pp. 572–575. DOI: 10.1016/S0140-6736(81)92029-8.

[149] Barry Halliwell and John M. C. Gutteridge. "The antioxidants of human extracellular fluids". In: *Archives of Biochemistry and Biophysics* 280.1 (July 1990), pp. 1–8. DOI: 10.1016/0003-9861(90)90510-6.

[150] Brooke L. Summers, Allan M. Rofe, and Peter Coyle. "Dietary Zinc Supplementation Throughout Pregnancy Protects Against Fetal Dysmorphology and Improves Postnatal Survival After Prenatal Ethanol Exposure in Mice". en. In: *Alcoholism: Clinical and Experimental Research* 33.4 (2009). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1530-0277.2008.00873.x, pp. 591–600. DOI: 10.1111/j.1530-0277.2008.00873.x.

[151] Luke C. Carey et al. "Ethanol decreases zinc transfer to the fetus in normal but not metallothionein-null mice". en. In: *Alcoholism: Clinical and Experimental Research* 24.8 (Aug. 2000), pp. 1236–1240. DOI: 10.1111/j.1530-0277.2000.tb02089.x.

[152] Luke C. Carey et al. “Maternal ethanol exposure is associated with decreased plasma zinc and increased fetal abnormalities in normal but not metallothionein-null mice”. en. In: *Alcoholism: Clinical and Experimental Research* 24.2 (Feb. 2000), pp. 213–219. DOI: 10.1111/j.1530-0277.2000.tb04593.x.

[153] Janet Y. Uriu-Adams et al. “Influence of copper on early development: prenatal and postnatal considerations”. en. In: *BioFactors* 36.2 (Mar. 2010), pp. 136–152. DOI: 10.1002/biof.85.

[154] Iqbal Hamza et al. “The metallochaperone Atox1 plays a critical role in perinatal copper homeostasis”. en. In: *Proceedings of the National Academy of Sciences* 98.12 (June 2001), pp. 6848–6852. DOI: 10.1073/pnas.111058498.

[155] Jaekwon Lee, Joseph R. Prohaska, and Dennis J. Thiele. “Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development”. en. In: *Proceedings of the National Academy of Sciences* 98.12 (June 2001), pp. 6842–6847. DOI: 10.1073/pnas.111058698.

[156] Yien-Ming Kuo et al. “The copper transporter CTR1 provides an essential function in mammalian embryonic development”. en. In: *Proceedings of the National Academy of Sciences* 98.12 (June 2001), pp. 6836–6841. DOI: 10.1073/pnas.111057298.

[157] Yoshinori Takahashi et al. “Mammalian Copper Chaperone Cox17p Has an Essential Role in Activation of Cytochrome *c* Oxidase and Embryonic Development”. en. In: *Molecular and Cellular Biology* 22.21 (Nov. 2002), pp. 7614–7621. DOI: 10.1128/MCB.22.21.7614-7621.2002.

[158] Gladys J. Everson, Huan-Chang Chow Tsai, and Tong-In Wang. “Copper Deficiency in the Guinea Pig”. en. In: *The Journal of Nutrition* 93.4 (Dec. 1967), pp. 533–540. DOI: 10.1093/jn/93.4.533.

[159] William W. Carlton and William A. Kelly. “Neural Lesions in the Offspring of Female Rats Fed Copper-deficient Diet”. en. In: *The Journal of Nutrition* 97.1 (Jan. 1969), pp. 42–52. DOI: 10.1093/jn/97.1.42.

[160] Cf Mills and Rb Williams. “Copper concentration and cytochrome-oxidase and ribonuclease activities in the brains of copper-deficient lambs”. en. In: *Biochemical Journal* 85.3 (Dec. 1962), pp. 629–632. DOI: 10.1042/bj0850629.

[161] B. Faye et al. “Copper deficiency in ruminants in the rift valley of east Africa”. en. In: *Tropical Animal Health and Production* 23.3 (Sept. 1991), pp. 172–180. DOI: 10.1007/BF02357001.

[162] J. R. Prohaska and W. W. Wells. “Copper Deficiency In The Developing Rat Brain: Evidence For Abnormal Mitochondria”. en. In: *Journal of Neurochemistry* 25.3 (Sept. 1975), pp. 221–228. DOI: 10.1111/j.1471-4159.1975.tb06956.x.

[163] Henriette S. Andersen et al. “Effect of dietary copper deficiency on iron metabolism in the pregnant rat”. en. In: *British Journal of Nutrition* 97.2 (Feb. 2007), pp. 239–246. DOI: 10.1017/S0007114507239960.

[164] Zivanit Ergaz et al. “The effect of copper deficiency on fetal growth and liver antioxidant capacity in the cohen diabetic rat model”. en. In: *Toxicology and Applied Pharmacology* 265.2 (Dec. 2012), pp. 209–220. DOI: 10.1016/j.taap.2012.10.006.

[165] Lorraine Gambling and Harry J. McArdle. “Iron, copper and fetal development”. en. In: *Proceedings of the Nutrition Society* 63.4 (Nov. 2004), pp. 553–562. DOI: 10.1079/PNS2004385.

[166] Iwona Lewicka et al. “Selected trace elements concentrations in pregnancy and their possible role — literature review”. In: *Ginekologia Polska* 88.9 (Sept. 2017), pp. 509–514. DOI: 10.5603/GP.a2017.0093.

[167] Molly E. Beckers-Trapp et al. “Abnormal development and increased 3-nitrotyrosine in copper-deficient mouse embryos”. en. In: *Free Radical Biology and Medicine* 40.1 (Jan. 2006), pp. 35–44. DOI: 10.1016/j.freeradbiomed.2005.08.020.

[168] Soo Jin Yang et al. “Effects of copper deficiency on mouse yolk sac vasculature and expression of angiogenic mediators”. en. In: *Birth Defects Research. Part B, Developmental and Reproductive Toxicology* 77.5 (Oct. 2006), pp. 445–454. DOI: 10.1002/bdrb.20096.

[169] Soo Jin Yang et al. “Low nitric oxide: a key factor underlying copper-deficiency teratogenicity”. en. In: *Free Radical Biology and Medicine* 43.12 (Dec. 2007), pp. 1639–1648. DOI: 10.1016/j.freeradbiomed.2007.08.031.

[170] Chandan K. Sen et al. “Copper-induced vascular endothelial growth factor expression and wound healing”. en. In: *American Journal of Physiology-Heart and Circulatory Physiology* 282.5 (May 2002), H1821–H1827. DOI: 10.1152/ajpheart.01015.2001.

[171] Lydia Finney et al. “COPPER AND ANGIOGENESIS: UNRAVELLING A RELATIONSHIP KEY TO CANCER PROGRESSION”. en. In: *Clinical and Experimental Pharmacology and Physiology* 36.1 (Jan. 2009), pp. 88–94. DOI: 10.1111/j.1440-1681.2008.04969.x.

[172] A Cordano. “Clinical manifestations of nutritional copper deficiency in infants and children”. en. In: *The American Journal of Clinical Nutrition* 67.5 (May 1998), 1012S–1016S. DOI: 10.1093/ajcn/67.5.1012S.

[173] Jack T. Saari et al. “Cardiac nitric oxide synthases are elevated in dietary copper deficiency”. en. In: *The Journal of Nutritional Biochemistry* 18.7 (July 2007), pp. 443–448. DOI: 10.1016/j.jnutbio.2006.07.006.

[174] Y James Kang et al. “Metallothionein Inhibits Myocardial Apoptosis in Copper-Deficient Mice: Role of Atrial Natriuretic Peptide”. en. In: *Laboratory Investigation* 80.5 (May 2000), pp. 745–757. DOI: 10.1038/labinvest.3780078.

[175] R. E. Wildman et al. “Marginal copper-restricted diets produce altered cardiac ultrastructure in the rat”. eng. In: *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* 210.1 (Oct. 1995), pp. 43–49. DOI: 10.3181/00379727-210-43923.

[176] Shunichi Shibazaki et al. "Copper deficiency caused by excessive alcohol consumption". en. In: *BMJ Case Reports* (Sept. 2017), bcr–2017–220921. DOI: 10.1136/bcr-2017-220921.

[177] Hongwei Lin et al. "Dietary Copper Plays an Important Role in Maintaining Intestinal Barrier Integrity During Alcohol-Induced Liver Disease Through Regulation of the Intestinal HIF-1 Signaling Pathway and Oxidative Stress". In: *Frontiers in Physiology* 11 (May 2020), p. 369. DOI: 10.3389/fphys.2020.00369.

[178] L. Glória et al. "Nutritional deficiencies in chronic alcoholics: relation to dietary intake and alcohol consumption". eng. In: *The American Journal of Gastroenterology* 92.3 (Mar. 1997), pp. 485–489.

[179] Cl Keen et al. "Effect of copper deficiency on prenatal development and pregnancy outcome". en. In: *The American Journal of Clinical Nutrition* 67.5 (May 1998), 1003S–1011S. DOI: 10.1093/ajcn/67.5.1003S.

[180] Charles S. Lieber. "Relationships Between Nutrition, Alcohol Use, and Liver Disease". In: *Alcohol Research & Health* 27.3 (2003), pp. 220–231.

[181] J.C. Bremert, I.E. Dreosti, and R.S. Tulsi. "A teratogenic interaction between dietary deficiencies of zinc and folic acid in rats: An electron microscope study". en. In: *Nutrition Research* 9.1 (Jan. 1989), pp. 105–112. DOI: 10.1016/S0271-5317(89)80108-3.

[182] Jong Ho Lee and F. L. Cerklewski. "Influence of Alcohol and Low Dietary Copper on Copper Utilization of Maternal and Offspring Liver". en. In: *Korean Journal of Nutrition* 23.6 (1990), pp. 443–450.

[183] Allison L Fisher and Elizabeta Nemeth. "Iron homeostasis during pregnancy". en. In: *The American Journal of Clinical Nutrition* 106 (Dec. 2017), 1567S–1574S. DOI: 10.3945/ajcn.117.155812.

[184] Thomas H Bothwell. "Iron requirements in pregnancy and strategies to meet them". en. In: *The American Journal of Clinical Nutrition* 72.1 (July 2000), 257S–264S. DOI: 10.1093/ajcn/72.1.257S.

[185] Nils Milman. "Iron and pregnancy—a delicate balance". en. In: *Annals of Hematology* 85.9 (Sept. 2006), pp. 559–565. DOI: 10.1007/s00277-006-0108-2.

[186] Clara Camaschella. "Iron-Deficiency Anemia". en. In: *New England Journal of Medicine* 372.19 (May 2015). Publisher: Massachusetts Medical Society _eprint: https://doi.org/10.1056/NEJMra1401038, pp. 1832–1843. DOI: 10.1056/NEJMra1401038.

[187] Raghavendra Rao and Michael K. Georgieff. "Iron in fetal and neonatal nutrition". In: *Seminars in Fetal and Neonatal Medicine*. Nutrition 12.1 (Feb. 2007), pp. 54–63. DOI: 10.1016/j.siny.2006.10.007.

[188] Jon F. R. Barrett et al. "Absorption of non-haem iron from food during normal pregnancy". en. In: *BMJ* 309.6947 (July 1994). Publisher: British Medical Journal Publishing Group Section: Paper, pp. 79–82. DOI: 10.1136/bmj.309.6947.79.

[189] Melissa F. Young et al. “Maternal Hepcidin Is Associated with Placental Transfer of Iron Derived from Dietary Heme and Nonheme Sources1–24”. In: *The Journal of Nutrition* 142.1 (Jan. 2012), pp. 33–39. DOI: 10.3945/jn.111.145961.

[190] Susanne van Santen et al. “The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study”. en. In: *Clinical Chemistry and Laboratory Medicine* 51.7 (July 2013). Publisher: De Gruyter Section: Clinical Chemistry and Laboratory Medicine (CCLM), pp. 1395–1401. DOI: 10.1515/cclm-2012-0576.

[191] Amat Bah et al. “Serum Hepcidin Concentrations Decline during Pregnancy and May Identify Iron Deficiency: Analysis of a Longitudinal Pregnancy Cohort in The Gambia1”. In: *The Journal of Nutrition* 147.6 (June 2017), pp. 1131–1137. DOI: 10.3945/jn/116.245373.

[192] K. N. Millard et al. “Changes in the expression of intestinal iron transport and hepatic regulatory molecules explain the enhanced iron absorption associated with pregnancy in the rat”. en. In: *Gut* 53.5 (May 2004). Publisher: BMJ Publishing Group Section: Small intestine, pp. 655–660. DOI: 10.1136/gut.2003.031153.

[193] Mari Rehu et al. “Maternal serum hepcidin is low at term and independent of cord blood iron status”. en. In: *European Journal of Haematology* 85.4 (2010). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1600-0609.2010.01479.x, pp. 345–352. DOI: 10.1111/j.1600-0609.2010.01479.x.

[194] Hannah Roberts, Stephane L. Bourque, and Stephen J. Renaud. “Maternal iron homeostasis: effect on placental development and function”. In: *Reproduction* 160.4 (Oct. 2020). Publisher: Bioscientifica Ltd Section: Reproduction, R65–R78. DOI: 10.1530/REP-20-0271.

[195] World Health Organization. *Daily iron and folic acid supplementation during pregnancy*. en. July 2024.

[196] João Ricardo Friedrisch and Bruno Kras Friedrisch. “Prophylactic iron supplementation in pregnancy: a controversial issue”. en. In: *Biochemistry Insights* 10 (Jan. 2017), p. 117862641773773. DOI: 10.1177/1178626417737738.

[197] Martin N. Mwangi, Andrew M. Prentice, and Hans Verhoef. “Safety and benefits of antenatal oral iron supplementation in low-income countries: a review”. en. In: *British Journal of Haematology* 177.6 (June 2017), pp. 884–895. DOI: 10.1111/bjh.14584.

[198] Nuria Aranda et al. “Pre-pregnancy iron reserves, iron supplementation during pregnancy, and birth weight”. en. In: *Early Human Development* 87.12 (Dec. 2011), pp. 791–797. DOI: 10.1016/j.earlhumdev.2011.06.003.

[199] Parul Christian et al. “Effects of prenatal micronutrient supplementation on complications of labor and delivery and puerperal morbidity in rural Nepal”. In: *International Journal of Gynecology & Obstetrics* 106.1 (July 2009), pp. 3–7. DOI: 10.1016/j.ijgo.2009.03.040.

[200] Domingo J. Piñero et al. “Variations in Dietary Iron Alter Brain Iron Metabolism in Developing Rats”. In: *The Journal of Nutrition* 130.2 (Feb. 2000), pp. 254–263. DOI: 10.1093/jn/130.2.254.

[201] John Beard, Keith M. Erikson, and Byron C. Jones. "Neonatal Iron Deficiency Results in Irreversible Changes in Dopamine Function in Rats". In: *The Journal of Nutrition* 133.4 (Apr. 2003), pp. 1174–1179. DOI: 10.1093/jn/133.4.1174.

[202] M. Roncagliolo et al. "Evidence of altered central nervous system development in infants with iron deficiency anemia at 6 mo: delayed maturation of auditory brainstem responses". en. In: *The American Journal of Clinical Nutrition* 68.3 (Sept. 1998), pp. 683–690. DOI: 10.1093/ajcn/68.3.683.

[203] Samantha McCann, Marta Perapoch Amadó, and Sophie E. Moore. "The role of iron in brain development: a systematic review". en. In: *Nutrients* 12.7 (July 2020), p. 2001. DOI: 10.3390/nu12072001.

[204] Victoria Arija et al. "Association of iron status and intake during pregnancy with neuropsychological outcomes in children aged 7 years: the prospective birth cohort infancia y medio ambiente (INMA) study". en. In: *Nutrients* 11.12 (Dec. 2019), p. 2999. DOI: 10.3390/nu11122999.

[205] C. Jayasinghe et al. "The effect of universal maternal antenatal iron supplementation on neurodevelopment in offspring: a systematic review and meta-analysis". en. In: *BMC Pediatrics* 18.1 (Dec. 2018), p. 150. DOI: 10.1186/s12887-018-1118-7.

[206] George N. Ioannou et al. "The effect of alcohol consumption on the prevalence of iron overload, iron deficiency, and iron deficiency anemia". en. In: *Gastroenterology* 126.5 (May 2004), pp. 1293–1301. DOI: 10.1053/j.gastro.2004.01.020.

[207] Duygu Dee Harrison-Findik. "Role of alcohol in the regulation of iron metabolism". en. In: *World Journal of Gastroenterology* 13.37 (2007), p. 4925. DOI: 10.3748/wjg.v13.i37.4925.

[208] Kris V. Kowdley. "Alcohol intake and iron overload: Another role for hepcidin?" en. In: *Hepatology* 45.2 (Feb. 2007), pp. 541–543. DOI: 10.1002/hep.21544.

[209] Echoleah S. Rufer et al. "Adequacy of maternal iron status protects against behavioral, neuroanatomical, and growth deficits in fetal alcohol spectrum disorders". en. In: *PLoS ONE* 7.10 (Oct. 2012). Ed. by Jonathan I. Matsui, e47499. DOI: 10.1371/journal.pone.0047499.

[210] R. Colin Carter et al. "Fetal Alcohol Exposure, Iron-Deficiency Anemia, and Infant Growth". en. In: *Pediatrics* 120.3 (Sept. 2007), pp. 559–567. DOI: 10.1542/peds.2007-0151.

[211] Kaylee K. Helfrich et al. "Gestational Iron Supplementation Improves Fetal Outcomes in a Rat Model of Prenatal Alcohol Exposure". en. In: *Nutrients* 14.8 (Apr. 2022), p. 1653. DOI: 10.3390/nu14081653.

[212] Canhua Huang and Yuanyuan Zhang, eds. *Oxidative Stress: Human Diseases and Medicine*. en. Singapore: Springer, 2021. DOI: 10.1007/978-981-16-0522-2.

[213] Zdeňka Ďuračková. "Free Radicals and Antioxidants for Non-Experts". en. In: *Systems Biology of Free Radicals and Antioxidants*. Ed. by Ismail Laher. Berlin, Heidelberg: Springer, 2014, pp. 3–38. DOI: 10.1007/978-3-642-30018-9_2.

[214] W. Bosron, T. Ehrig, and T.-K. Li. "Genetic Factors in Alcohol Metabolism and Alcoholism". en. In: *Seminars in Liver Disease* 13.02 (May 1993), pp. 126–135. DOI: 10.1055/s-2007-1007344.

[215] Hirokazu Yokoyama, Enrique Baraona, and Charles S Lieber. "Molecular Cloning and Chromosomal Localization of the ADH7 Gene Encoding Human Class IV (s) ADH". en. In: *Genomics* 31.2 (Jan. 1996), pp. 243–5.

[216] Samir Zakhari. "Overview: How Is Alcohol Metabolized by the Body?" en. In: *Alcohol Res Health* 29.4 (2006), pp. 245–54.

[217] Arthur I. Cederbaum. "Alcohol Metabolism". en. In: *Clinics in Liver Disease* 16.4 (Nov. 2012), pp. 667–685. DOI: 10.1016/j.cld.2012.08.002.

[218] Chloé Hugbart et al. "Non-oxidative ethanol metabolism in human hepatic cells in vitro: Involvement of uridine diphospho-glucuronosyltransferase 1A9 in ethylglucuronide production". en. In: *Toxicology in Vitro* 66 (Aug. 2020), p. 104842. DOI: 10.1016/j.tiv.2020.104842.

[219] Anders Helander and Olof Beck. "Ethyl Sulfate: A Metabolite of Ethanol in Humans and a Potential Biomarker of Acute Alcohol Intake". en. In: *Journal of Analytical Toxicology* 29.5 (July 2005), pp. 270–274. DOI: 10.1093/jat/29.5.270.

[220] Bhupendra S. Kaphalia et al. "Fatty acid ethyl esters: markers of alcohol abuse and alcoholism". en. In: *Alcohol* 34.2-3 (Oct. 2004), pp. 151–158. DOI: 10.1016/j.alcohol.2004.07.013.

[221] Sharmistha Chatterjee and Parames C. Sil. "ROS-Influenced Regulatory Cross-Talk With Wnt Signaling Pathway During Perinatal Development". In: *Frontiers in Molecular Biosciences* 9 (Apr. 2022), p. 889719. DOI: 10.3389/fmolb.2022.889719.

[222] Toren Finkel. "Signal transduction by reactive oxygen species". In: *The Journal of Cell Biology* 194.1 (July 2011), pp. 7–15. DOI: 10.1083/jcb.201102095.

[223] Yijiang Zhou et al. "Reactive oxygen species in vascular formation and development". en. In: *Oxidative Medicine and Cellular Longevity* 2013 (2013), pp. 1–14. DOI: 10.1155/2013/374963.

[224] Alexandra Almansa-Ordonez et al. "Oxidative stress in reproduction: a mitochondrial perspective". en. In: *Biology* 9.9 (Sept. 2020), p. 269. DOI: 10.3390/biology9090269.

[225] Shama Bhatia et al. "Oxidative stress and DNA damage in the mechanism of fetal alcohol spectrum disorders". en. In: *Birth Defects Research* 111.12 (July 2019), pp. 714–748. DOI: 10.1002/bdr2.1509.

[226] Peter G. Wells et al. "Oxidative Stress in Developmental Origins of Disease: Teratogenesis, Neurodevelopmental Deficits, and Cancer". en. In: *Toxicological Sciences* 108.1 (Mar. 2009), pp. 4–18. DOI: 10.1093/toxsci/kfn263.

[227] Jack Uetrecht, Denis M. Grant, and Peter G. Wells. "Biochemical mechanisms of drug toxicity". en. In: *Atkinson's Principles of Clinical Pharmacology*. Elsevier, 2022, pp. 267–302. DOI: 10.1016/B978-0-12-819869-8.00015-X.

[228] Ginevra Micangeli et al. “The Impact of Oxidative Stress on Pediatrics Syndromes”. en. In: *Antioxidants* 11.10 (Oct. 2022), p. 1983. DOI: 10.3390/antiox11101983.

[229] Gerald Thomas et al. “The effectiveness of alcohol warning labels in the prevention of Fetal Alcohol Spectrum Disorder: A brief review”. In: *The International Journal of Alcohol and Drug Research* 3.1 (Mar. 2014), pp. 91–103. DOI: 10.7895/ijadr.v3i1.126.

[230] Janet R. Hankin. “Fetal alcohol syndrome prevention research”. en. In: *Alcohol Research & Health* 26.1 (2002), p. 58.

[231] William R. Miller and Gary S. Rose. “Toward a theory of motivational interviewing.” en. In: *American Psychologist* 64.6 (2009), pp. 527–537. DOI: 10.1037/a0016830.

[232] R. Louise Floyd et al. “Preventing alcohol-exposed pregnancies: a randomized controlled trial”. en. In: *American Journal of Preventive Medicine* 32.1 (Jan. 2007), pp. 1–10. DOI: 10.1016/j.amepre.2006.08.028.

[233] Karen S. Ingersoll et al. “Preconceptional motivational interviewing interventions to reduce alcohol-exposed pregnancy risk”. In: *Journal of Substance Abuse Treatment* 44.4 (Apr. 2013), pp. 407–416. DOI: 10.1016/j.jsat.2012.10.001.

[234] Katalin Ujhelyi Gomez et al. “Are psychosocial interventions effective in reducing alcohol consumption during pregnancy and motherhood? A systematic review and meta-analysis”. en. In: *Addiction* 116.7 (2021). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/add.15296, pp. 1638–1663. DOI: 10.1111/add.15296.

[235] Svetlana Popova et al. “Effectiveness of brief alcohol interventions for pregnant women: a systematic literature review and meta-analysis”. en. In: *BMC Pregnancy and Childbirth* 23.1 (Jan. 2023), p. 61. DOI: 10.1186/s12884-023-05344-8.

[236] May Na Erng, Andrew Smirnov, and Natasha Reid. “Prevention of Alcohol-Exposed Pregnancies and Fetal Alcohol Spectrum Disorder Among Pregnant and Postpartum Women: A Systematic Review”. en. In: *Alcoholism: Clinical and Experimental Research* 44.12 (2020). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/acer.14489, pp. 2431–2448. DOI: 10.1111/acer.14489.

[237] G Chang. “Reducing Prenatal Alcohol Exposure and the Incidence of FASD: Is the Past Prologue?” In: *Alcohol Research: Current Reviews* 43.1 (2023), p. 02. DOI: 10.35946/arcr.v43.1.02.

[238] Lyndsay Ammon Avalos et al. “Does Lack of Multinutrient Supplementation During Early Pregnancy Increase Vulnerability to Alcohol-Related Preterm or Small-for-Gestational-Age Births?” en. In: *Maternal and Child Health Journal* 15.8 (Nov. 2011), pp. 1324–1332. DOI: 10.1007/s10995-010-0690-8.

[239] Yuanpei Zhang et al. “A review of interventions against fetal alcohol spectrum disorder targeting oxidative stress”. en. In: *International Journal of Developmental Neuroscience* 71.1 (Dec. 2018). 00011, pp. 140–145. DOI: 10.1016/j.ijdevneu.2018.09.001.

Chapter 2

Novel γ-glutamyl-cysteine Compounds and Oxidative Damage in a Rat Model of Marginal Zinc Deficiency.

2.1 Introduction

GSH is one of the most important antioxidants in mammalian cells. Studies have shown that GSH is effective in helping to reduce oxidative stress in conditions such as diabetes, obesity, liver dysfunction, and inflammation[1,2]. Excessive oxidative stress can cause oxidative damage leading to DNA damage, lipid peroxidation, protein damage, and can contribute to the progression and pathology of diseases such as cancer and cardiovascular disease[3]. This study investigated the extent to which oral supplementation of a glutathione precursor can increase the oxidative defense system and reduce the damage caused by zinc deficiency-induced ROS.

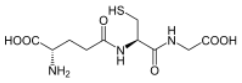
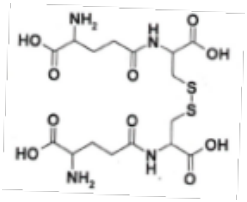
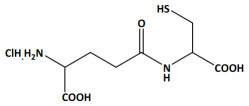
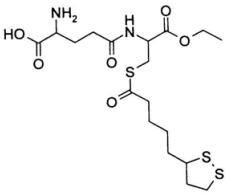
Most cells in the human body are able to synthesize GSH from glutamate, cysteine, and glycine. In the first and rate-limiting step, γ-glutamyl-cysteine (GGC) is synthesized from L-glutamate and L-cysteine by γ-glutamyl-cysteine synthetase (GCL). In the second

step, glycine is conjugated to the C-terminus of GGC by GSH synthetase (GS), producing GSH[4]. Intracellularly, GSH exists in both the reduced (GSH) state and the oxidized disulfide form (oxidized glutathione (GSSG)). The ratio between GSH and GSSG is held in dynamic balance depending on factors such as intracellular damage and intracellular demand for oxidative defense[5].

Studies determining the actual bioavailability and metabolic fate of oral supplementation of GSH have led to mixed results. Some human studies have shown that an oral administration of GSH for four weeks, even at high doses, did not lead to a significant change in blood GSH levels[5]. Other studies, which looked at the longer term and immediate effects of GSH supplementation, saw an 35% increase in blood GSH levels after six months of supplementation and a significant increase in protein-bound GSH one to two hours after GSH supplementation[4,6]. Supplementation with GSH precursors, such as N-acetylcysteine (NAC) or sodium salts of GSH, have been shown to benefit patients with diabetes[7,8].

The current study examines whether oxidative stress and damage induced by marginal zinc deficiency (MZD) can be reduced by the supplementation of novel glutathione precursors of GGC: GGC-Bis (GGC-Bis), GGC-Hydrochloride (GGC-HCL), and GGC-Lipoic acid conjugate (GGC-La) in a dose-dependent manner (Table 2.1). There is no current information regarding the effectiveness of supplementation with these novel compounds or how effective they are compared to supplementation with glutathione itself. We hypothesize that supplementation with these novel GGC compounds will increase glutathione levels in blood and tissues, decrease markers of oxidative damage and increase markers of oxidative defense capacity.

Table 2.1: Three novel gamma-glutamylcysteine (GGC) compounds and glutathione were tested.

Compound Name	Molecular Formula	Molecular Weight (g/mol)	Structure
Glutathione	$C_{10}H_{17}N_3O_6S$	307.32	
GGC-Bis	$C_{16}H_{26}N_4O_{10}S_2$	498.53	
GGC-HCL	$C_8H_{15}ClN_2O_5S$	286.73	
GGC-La	$C_{18}H_{30}N_2O_6S_3$	466.64	

2.2 Materials and Methods

2.2.1 Animals

Weanling male CD rats (N=100; Charles River, Portage, MI) were acclimated to a semi-purified egg white-based zinc adequate Control diet (25 µg Zn/g diet; zinc adequate Control (ZnA Control)) and water *ad libitum* for one week in our facility[9]. After this acclimation period, rats were housed in individual stainless steel wire-bottomed cages in a room maintained at 22 °C to 25 °C with a 12-hour light:dark cycle and randomly assigned to one of ten experimental groups (N=10/group). All animal procedures and handling were conducted in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines.

2.2.2 Experimental Design

Rats in eight experimental groups consumed a MZD diet containing 5 µg Zn/g diet and supplemented with either a high dose (58.3 mg/day) or low dose (2.92 mg/day) of one of three novel GGC compounds or glutathione. The high dose was based on a 2000 mg/day of glutathione human equivalent, which was previously shown to significantly increase glutathione levels in erythrocytes[6]. The low dose represented a human equivalent of 500 mg/day. Calculations for rat dosages and dietary concentrations were based on a 60 kg man, using a human to rat conversion factor of 7, and dietary intake data showing a 250 g rat eating an average of approximately 25 g/day[10]. Rats in the remaining two experimental groups were either fed the MZD diet alone or the ZnA Control diet. All rats were fed their diets for 21 days during which 3-day food intake data was measured and body weights were recorded every third day. On the morning of day 22, rats were euthanized with isoflurane and blood and tissue were collected. All tissues not used for same-day analyses were frozen in liquid nitrogen and stored at −80 °C for future analyses.

2.2.3 Liver and Erythrocyte Glutathione Assay

After euthanization, livers were rapidly removed, weighed, rinsed in MES buffer (2-(N-morpholino)ethanesulfonic acid) and kept on ice. A 0.5 g portion of liver was homogenized in ice-cold MES buffer and deproteinized by the addition of an equal volume of metaphosphoric acid (MPA). Blood was collected in lithium heparinized tubes and erythrocytes were separated from the plasma and buffy coat by centrifugation at 1000 ×g for 10 min at 4 °C. A 1 mL aliquot of crude erythrocytes was lysed by the addition of 4 mL of ice cold water. After centrifugation at 10 000 ×g for 15 min at 4 °C, 2 mL of the supernatant was deproteinized by the addition of 2 mL of MPA. Both liver and erythrocyte samples were then stored at −20 °C until they were analyzed using a Glutathione Assay Kit for total glutathione and GSSG measurements. Reduced glutathione (GSH) concentrations were calculated as the difference between total glutathione and GSSG.

2.2.4 Erythrocyte Hemolysis Assay

We used a modified method from Zhu et al. (2005) to assess the susceptibility of erythrocytes to oxidant stress-induced hemolysis. Erythrocytes were separated from plasma and buffy coat by centrifugation at 1800 ×g for 15 min at 4 °C. The crude erythrocyte pellets were washed with phosphate-buffered saline (PBS) and centrifuged at 1800 ×g for 5 min. This procedure was repeated four times. During the last wash, erythrocytes were centrifuged at 1800 ×g for 15 min at 4 °C. The packed erythrocytes (400 µL) were then suspended in 600 µL of PBS[11].

Erythrocytes were resuspended in PBS to achieve a final hematocrit of 3 %. Oxidative stress was induced by the addition of 200 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) to the erythrocyte sample and the tubes were incubated at 37 °C. Every 7.5 min, 125 µL of the samples were obtained and added to 1 mL of either water (total hemolysis control for the timepoint) or PBS. After centrifugation at 3000 ×g for 5 min at 4 °C, the hemoglobin concentration in the supernatants were determined by measuring the absorbance at 540 nm. The percent of hemolysis induced by AAPH was calculated by the equation:

$$\% \text{ Hemolysis} = \left[\frac{H_{\text{PBS}}}{H_{\text{Water}}}\right] \times 100 \tag{2.1}$$

where H_{PBS} is the absorbance of the sample added to 1 mL of PBS and H_{Water} is the absorbance of the sample added to 1 mL of water (total hemolysis control).

2.2.5 Plasma TBARS Assay

Plasma TBARS were assessed as an index of lipid peroxidation as we have previously described[12]. Briefly, 100 µL of plasma was combined with 200 µL of 3 % sodium dodecyl sulfate, 800 µL of 0.1 mol L^{-1} HCl, 100 µL of 10 % (wt/vol) phosphotungstic acid, and 400 µL of 0.7 % (wt/vol) 2-thiobarbituric acid (TBA), vortex-mixed, and incubated at 95 °C for 30 min. Samples were cooled on ice and mixed with 1 mL of 1-butanol. After centrifugation (1800 g, 10 min, 4 °C), 200 µL of the butanol phase was analyzed spectrofluorometrically

(excitation; 515 nm, emission; 555 nm) using a plate reader (Cetus, Perkin-Elmer, Norwalk, CT). TBARS was expressed as malondialdehyde (MDA) equivalents.

2.2.6 Statistical Analysis

Statistical analyses were performed using SPSS Statistics 23, SAS and Statview. One-way analysis of variance (ANOVA) were used to determine significance of effects. Two sets of Dunnett comparisons were performed to determine differences in the treatments groups from either the ZnA Control or the MZD group.

Erythrocyte hemolysis data (sigmoidal over time for the kinetics assay) were floored and capped (0,100) and logit transformed as follows:

$$\text{logit}(y) = \log\left(\frac{\text{pct} + 1}{100 - \text{pct} + 1}\right) \tag{2.2}$$

The transformation allowed linearization of the sigmoidal data. Treatment data were paired to ZnA Control data for each day, and the lines averaged over the dates. The difference in slopes of the logit curves among the treatments was evaluated as an index of erythrocyte hemolysis response. Erythrocyte hemolysis data were also analyzed by assessing the estimated time it took to reach 50% hemolysis (time to 50% hemolysis (LT50)). A Shapiro-Wilk test was used to determine normality. A p-value <0.05 was considered significant. Data are presented as means(SEM).

2.3 Results

2.3.1 Body Weight and Percent Change in Body Weight

Body weight was measured every three days starting with Day 0 and ending on Day 21, the day before rats were euthanized (Table 2.2). Body weights were similar among the groups up to Day 15. However, by Day 18, animals on the MZD diet and most of the animals on

the MZD + test compound diets weighed less than the ZnA Control animals; only animals on the MZD+GGC-HCL High and MZD+GGC-La Low diets had body weights that were not significantly different from ZnA Control rats. By Day 21, animals fed the MZD diet were approximately 30 g lighter than ZnA Control rats. Only rats fed the MZD+GGC-La Low diet had similar body weights as the ZnA Control on Day 21.

The percent change in body weight (relative to Day 0) was not different among the groups until Day 9 (Table 2.3). ZnA Control animals had a higher percent change in body weight than all other groups starting from Day 3, indicating normal growth in these animals (Figure 2.1).

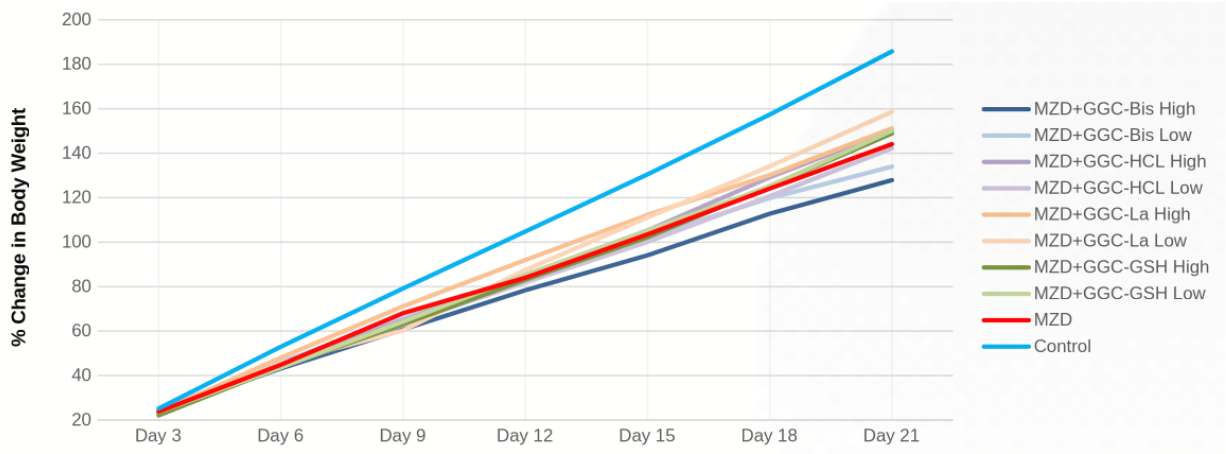


Figure 2.1: Percent change in body weight (relative to Day 0) over 21 days.

2.3.2 Food Intake and Food Efficiency Ratio

With regard to 3-day food intake (Table 2.4), on Day 3, animals fed the MZD diet did not differ from animals on the ZnA Control diet. However, by Day 6, MZD animals had significantly lower 3-day food intake ((46.3 ± 1.4) g) than the ZnA Control animals ((51.5 ± 1.5) g). This difference continued to increase up to Day 21, with the MZD animals consuming 16 % less diet than ZnA Control animals during the last three days of the study. At Day 21, rats fed the MZD diets supplemented with GGC-Bis (High and Low dose), GGC-La High dose, and GSH Low dose had significantly lower food intake compared to ZnA Control.

Food efficiency ratio was calculated by dividing 3-day body weight gain (g) by 3-day food intake (g) (Table 2.5). On Day 6, animals fed the MZD, MZD+GGC-Bis High, MZD+GGC-Bis Low, MZD+GGC-HCL Low, and MZD+GSH Low diets had significantly lower food efficiency ratios than rats fed the ZnA Control diet. However, by Day 21, only rats fed the MZD+GGC-Bis High and MZD+GGC-Bis Low had significantly lower food efficiency ratios compared to ZnA Control animals.

2.3.3 Tissue Weight and Relative Tissue Weight

Liver, kidney, testes, heart, and spleen weights are shown in Table 2.6. Rats fed the MZD+GGC-Bis diets had significantly higher liver weights than ZnA Control when expressed per organ weight but not when expressed relative to body weight (Table 2.7). Livers from the MZD+GGC-La Low and High groups had higher relative tissue weight than ZnA Control. Rats fed the ZnA Control diet had significantly higher kidney weights than all other groups except for MZD+GGC-HCL Low and MZD+GGC-La Low diets; however, these differences were no longer significant when expressed relative to body weight. There were no differences among groups with regard to testes weights; however, when expressed relative to body weight, all groups except MZD+GGC-La Low and MZD+GSH Low had higher relative testes weight compared to ZnA Control rats. Similar to kidney weights, ZnA Control rats had significantly higher spleen weights than all other groups except for MZD+GGC-HCL Low and MZD+GGC-La Low diets; these differences were no longer significant when spleen weights were expressed relative to body weight. Heart weights were similar among the groups whether expressed on an organ weight basis or relative to body weight.

2.3.4 Total Glutathione, GSH, GSSG, and GSH:GSSG in Liver and Erythrocytes

Total Glutathione, GSH, and GSH:GSSG ratios in liver and erythrocytes are shown in Table 2.8. Although the ZnA Control and MZD groups were not significantly different, rats fed MZD+GGC-HCL (High and Low dose) had significantly higher total liver glutathione and liver GSH concentrations than both the ZnA Control and MZD groups. Animals fed MZD+GGC-La High had significantly lower liver GSSG concentrations than all other groups, and animals fed MZD+GGC-La Low had significantly lower erythrocyte GSH concentrations than all other groups. The concentrations of total glutathione and GSSG in erythrocytes and the GSH:GSSG ratios in the liver and erythrocytes were not significantly different among treatment groups.

2.3.5 TBARS

The concentrations of TBARS (µM MDA) were not significantly different among treatment groups (Table 2.9).

2.3.6 Erythrocyte Hemolysis

Looking at just the ZnA Control and MZD groups, ANOVA performed on the raw kinetic data showed that the MZD group had a significantly higher percentage of erythrocyte hemolysis compared to the ZnA Control group at 30 min ($p < 0.05$) (Table 2.10, Figure 2.2). Similarly, when data were floored, capped and logit transformed, the estimated time to reach 50% hemolysis (LT50) was significantly sooner for the MZD group versus the ZnA Control group ($p = 0.0366$), while the difference in slopes (erythrocyte hemolysis response) was marginally significant ($p = 0.0785$). However, when all of the treatment groups were included, there were no significant differences among any of the groups when analyzed as raw data or logit transformed data using ANOVA. Similarly, there were no differences among the groups when

Dunnett comparisons were performed.

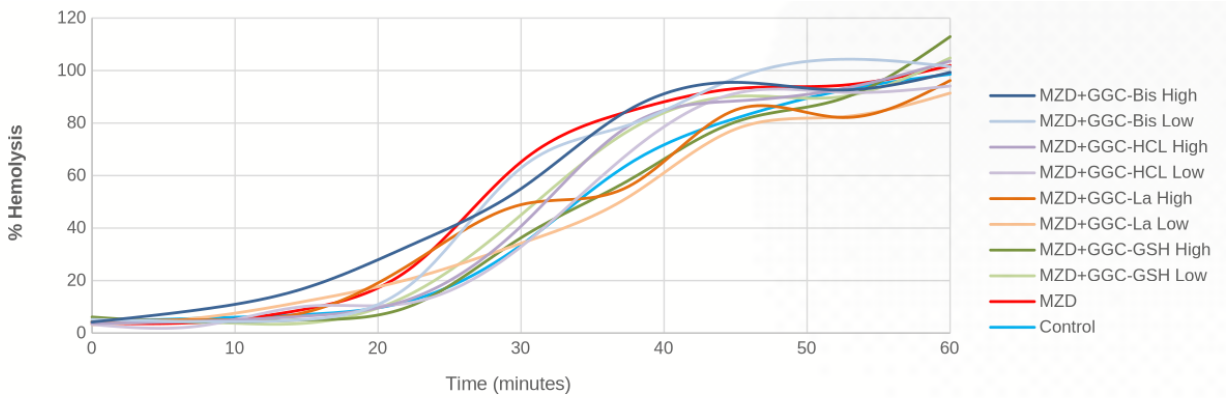


Figure 2.2: Erythrocyte hemolysis kinetics (% hemolysis over time) following AAPH-induced oxidative stress.

2.4 Discussion

This study tested whether novel γ-glutamyl-cysteine (GGC) compounds could reduce oxidative damage from (MZD). The MZD diet successfully induced a state of oxidative stress, demonstrated by the increased fragility of erythrocytes to an oxidative challenge. This functional outcome occurred without corresponding changes in plasma lipid peroxidation or baseline glutathione concentrations in the liver and erythrocytes. Supplementation with the novel GGC compounds or glutathione did not confer significant protection against this MZD-induced erythrocyte fragility. Certain compounds did increase glutathione concentrations in the liver and erythrocytes, suggesting bioavailability and synthesis into glutathione.

2.4.1 Oxidative Stress Induced by Zinc Deficiency

Zinc deficiency can lead to increased levels of ROS and subsequent oxidative stress which can damage DNA, proteins, and lipids. The source of oxidative stress from zinc deficiency may include decreased levels of zinc-dependent antioxidants such as CuZnSOD and zinc-MT, and disrupted function and expression of the mitochondrial electron transport chain compo-

nents. Zinc deficiency has also been associated with protein misfolding in the endoplasmic reticulum (ER) and increased NOXs activity, both of which are associated with increased ROS production. Zinc is also an integral component of the phospholipid bilayer of membranes. When zinc is low, redox active metals, such as iron and copper, can replace zinc in the membrane. Without redox-inactive zinc, iron and copper can catalyze the generation of radicals and induce lipid peroxidation leading to increased cell membrane fragility[13].

Previous research has shown that zinc deficiency can affect glutathione synthesis by modulating the enzyme involved in the first step of glutathione synthesis (GCL) at the transcriptional and posttranslational levels[10]. GCL, the catalyst for the rate limiting step in glutathione synthesis, is regulated by transcription factors Nuclear factor erythroid 2-related factor 2 (Nrf2), Activator Protein-1 (AP-1), and NF-κB. The combination of Nrf2's zinc-dependent activation, zinc deficiency-induced inhibition of NF-κB activation, and activation of caspase 3, a zinc enzyme that cleaves the GCL catalytic subunit, has led to zinc deficiency being associated with GSH deficiency in various cells and tissues[13]..

2.4.2 Food Intake and Body/Tissue Weights

As expected, MZD decreased food intake and body weight in our study. Suboptimal zinc intake is particularly detrimental when instituted early in development, e.g., weanling animals[14]. After normalizing to body weight, only the MZD groups that were supplemented with MZD+GGC-La (both High and Low doses), had higher liver/body weight ratios compared to ZnA Control. These livers appeared to be normal on gross examination during dissection and their increased size may be due to a compensatory response to the MZD diet and/or the GGC-La compound.

2.4.3 TBARS

In some but not all studies, zinc deficiency increases lipid peroxidation as assessed by TBARS measurement[15]. In an ethanol-exposed piglet model, chronic oxidative stress alters the

polyunsaturated profiles, e.g., reduces polyunsaturated fatty acid (PUFA) synthesis which can result in lower TBARS as there are fewer PUFAs to peroxidize over time[16]. It is possible that the zinc deficiency regimen employed in this study also changed PUFA profiles such that the lipid peroxidizability index was lower in MZD compared to ZnA Control.

2.4.4 Liver and Erythrocyte Glutathione

As previously mentioned, zinc deficiency can have a negative effect on the tissue levels of glutathione. Studies looking at the effects of severe zinc deficiency (1 µg Zn/g diet) on glutathione levels reported significant decreases in the concentrations of glutathione in the liver, kidney, and plasma[17,18]. Because zinc may affect glutathione levels by modulating GCL, the rate-limiting enzyme in GGC synthesis, supplementation with novel GGC compounds could bypass zinc deficiency's effects and increase glutathione levels.

We did not observe any significant differences in total glutathione, GSSG, or GSH levels in either the liver nor erythrocytes between the ZnA Control and MZD groups. Although both MZD+GGC-HCL High and Low dose groups had significantly higher liver total glutathione and GSH concentrations compared to ZnA Control, they did not differ from MZD animals. The MZD+GGC-La Low dose group had the highest erythrocyte concentration of total glutathione; however, it was not statistically significant ($p = 0.0812$). Interestingly, the same group also had significantly higher erythrocyte GSH concentrations than both the ZnA Control and MZD groups. This may help explain, in part, the fact that the MZD+GGC-La group had kinetic curves most similar to ZnA Control in the erythrocyte hemolysis assay.

2.4.5 Erythrocyte Hemolysis

Numerous reports show that oxidative stress conditions such as diabetes and alcohol exposure increase oxidative damage to circulating erythrocytes and result in an increased fragility of the erythrocyte membrane. In our study, MZD for three weeks resulted in a significant increase in erythrocyte hemolysis compared to ZnA Control when erythrocytes were exposed

to an oxidative stress condition *in vitro*. This increased susceptibility to oxidative stress is a functional endpoint and indicates that the marginal zinc deficiency was effective in inducing an oxidative stress condition. The differences between the MZD and ZnA Control erythrocyte hemolysis was observed with raw data, transformed data, and LT50 data when the ZnA Control and MZD groups were compared directly, but not when compared with all 10 treatment groups. There were no statistically significant effects of any of the GGC test compounds or glutathione supplement on reducing oxidative stress; however, MZD+GGC-La groups had hemolysis kinetic curves most similar to the ZnA Control.

Erythrocytes have several membrane defense systems to protect themselves from oxidative damage and hemolysis, including intracellular SOD, GSH, GPx, and catalase. Scavengers which are lipid-soluble, such as α-tocopherol, play a vital role by scavenging radicals within the lipid region of the membrane[19]. However, while water-soluble antioxidants such as Vitamin C and glutathione may act as a first defense when radicals are generated in the plasma, they are less effective at scavenging radicals within the membranes. Vitamin C has previously been shown to be less effective at preventing erythrocyte hemolysis[20]. Because erythrocytes are washed with PBS before incubation with the oxidant generator *in vitro*, any antioxidant defense which could potentially be provided by circulating GSH or GGC in the plasma would not be present. It is possible that if the erythrocytes had been incubated with plasma from each of the treated animals, there may have been additional protection from oxidative stress-induced hemolysis.

2.4.6 Role of Sulfur Amino Acids in Mitigating Oxidative Stress

Zinc deficiency leads to increased erythrocyte fragility in part due to increased lipid peroxidation by redox active metals bound to the erythrocyte membrane. Interestingly, the amount of sulfur amino acids in the diet can impact oxidative stress. Sulfur amino acids, like GSH, can act as free radical quenchers. By absorbing a free radical hit, the sulfhydryl group is oxidized and in the process, can protect macromolecules from oxidative damage.

In a study by Li et al. (2002), protein malnourished CD-1 mice fed cysteine supplemented diets restored their GSH levels and redox status (GSH:GSSG) in liver tissue[21]. A study by O'Dell et al. (1987) showed that supplementation of a 20 % soy protein diet with a 0.9 % methionine, giving it a sulfur amino acid level comparable to a 20 % egg white diet, reduced the osmotic fragility of erythrocyte membranes induced by incubation in a hypotonic solution of animals fed a zinc deficient diet ($< 1\,\mu g$ Zn/g)[22]. While O'Dell's study did not test whether the same effect could be seen with erythrocyte hemolysis induced with a free radical generator, it is possible that the sulfur-containing amino acids in our study diet ameliorated some of the oxidative stress caused by MZD.

2.5 Conclusion

MZD reduced body weight and food intake, indicating a metabolic effect of suboptimal zinc status. Data from this study also showed significant differences between ZnA Control and MZD animals in the erythrocyte hemolysis assay, an important functional endpoint. We did not observe differences between MZD and ZnA Control with regard to lipid peroxidation measurements or GSH:GSSG ratios, which give an indication of the cellular redox state. Our aim was to produce an oxidative stress condition that was measurable but not so severe that it would be overwhelming. In our pilot studies, we evaluated the effects of severe zinc deficiency (SZD) (1 µg Zn/g diet), MZD (5 µg Zn/g diet), and a zinc adequate (ZnA) diet (25 µg Zn/g diet) in weanling animals. By Day 21, animals fed the SZD diet had 50 % reductions in body weights and markedly depressed food intake compared to rats fed the ZnA diet. Because of this, we determined that the negative effects of SZD were too deleterious to be appropriate to test the effectiveness of a dietary antioxidant.

In a second pilot study, we observed significant differences between ZnA Control and MZD erythrocyte hemolysis rates after animals were fed their respective diets for 21 days (but not for 14 days). Therefore, we hypothesized that significant differences in other oxidative stress

and damage indices (TBARS and GSH:GSSG) would be observed as well. It is possible that greater oxidative stress and damage would occur had the study been extended for a longer period of time.

In conclusion, supplementation with three novel GGC compounds or glutathione did not significantly improve zinc deficiency-induced reductions in oxidative stress and damage as assessed by the erythrocyte hemolysis assay. In the future, we suggest feeding animals test diets for more than 21 days, observing plasma GSH levels, incubating erythrocytes in plasma during the erythrocyte hemolysis assay, and analyzing TBARS in testes, a tissue that is particularly susceptible to zinc deficiency-induced oxidative stress.

Table 2.2: Body weight (grams) of rats over 21 days across different dietary groups.

	Body Weight (g)							
Diet	**Day 0**	**Day 3**	**Day 6**	**Day 9**	**Day 12**	**Day 15**	**Day 18**	**Day 21**
ZnA Control	98.9 ± 3.7	123.6 ± 4.3	150.9 ± 5.4	176.4 ± 6.3	201.5 ± 7.3	226.5 ± 8.0	253.2 ± 9.8^{a}	281.1 ± 10.9^{a}
MZD+GGC-Bis High	100.2 ± 3.2	124.1 ± 3.5	143.1 ± 3.9	160.9 ± 5.1	178.3 ± 5.2	194.1 ± 6.2	212.9 ± 6.6^{b}	228.0 ± 7.3^{b}
MZD+GGC-Bis Low	100.9 ± 2.7	125.4 ± 2.5	146.8 ± 2.5	166.2 ± 3.3	185.7 ± 3.0	202.6 ± 4.2	220.1 ± 4.9^{bc}	234.0 ± 5.2^{bd}
MZD+GGC-HCL High	103.6 ± 1.8	126.8 ± 2.3	150.4 ± 2.9	170.6 ± 3.4	191.6 ± 3.2	212.4 ± 3.3	237.3 ± 4.2^{ac}	259.2 ± 4.1^{c}
MZD+GGC-HCL Low	104.7 ± 1.8	128.5 ± 2.5	150.6 ± 3.4	171.0 ± 4.2	190.5 ± 4.4	209.8 ± 5.7	231.4 ± 7.4^{bc}	253.9 ± 7.7^{cd}
MZD+GGC-La High	98.7 ± 3.8	121.8 ± 4.5	145.4 ± 4.2	168.0 ± 4.5	188.0 ± 4.4	207.5 ± 5.2	224.7 ± 4.9^{bc}	245.1 ± 4.8^{bc}
MZD+GGC-La Low	101.4 ± 3.8	123.9 ± 4.0	147.5 ± 5.1	162.2 ± 8.2	190.0 ± 7.4	214.1 ± 9.0	237.6 ± 10.2^{ac}	262.1 ± 10.8^{ac}
MZD+GSH High	101.3 ± 3.4	123.8 ± 4.3	145.6 ± 4.6	165.3 ± 5.9	185.7 ± 6.9	204.9 ± 7.4	228.0 ± 8.8^{bc}	252.4 ± 10.3^{cd}
MZD+GSH Low	101.1 ± 3.5	124.6 ± 3.7	144.5 ± 3.3	165.2 ± 3.6	186.3 ± 3.9	206.1 ± 4.2	226.3 ± 5.0^{bc}	251.5 ± 5.6^{cd}
MZD	101.9 ± 3.5	126.0 ± 3.8	147.3 ± 4.5	170.7 ± 4.8	186.8 ± 5.2	206.6 ± 5.6	227.9 ± 7.2^{bc}	247.8 ± 6.9^{bc}
ANOVA P-value	0.9621	0.9789	0.9107	0.6021	0.2900	0.0645	0.0204	0.0006

Values are presented as mean ± SEM. Within a column, values not sharing a common superscript letter (a, b, c, d) are significantly different ($p < 0.05$).

Table 2.3: Percent change in body weight relative to Day 0 (%).

	% Change in Body Weight						
Diet	**Day 3**	**Day 6**	**Day 9**	**Day 12**	**Day 15**	**Day 18**	**Day 21**
ZnA Control	25.2 ± 1.9	53.0 ± 3.4	79.2 ± 5.2^{a}	104.8 ± 6.5^{a}	130.5 ± 7.6^{a}	157.4 ± 8.6^{a}	185.8 ± 10.0^{a}
MZD+GGC-Bis High	24.1 ± 0.9	43.2 ± 1.9	60.8 ± 2.0^{bc}	78.4 ± 3.0^{b}	94.1 ± 3.3^{b}	112.8 ± 2.2^{b}	127.9 ± 3.5^{b}
MZD+GGC-Bis Low	24.6 ± 1.3	46.1 ± 3.1	65.7 ± 4.9^{bc}	85.3 ± 5.9^{bc}	102.2 ± 7.3^{bc}	119.8 ± 8.9^{bc}	134.0 ± 10.5^{bc}
MZD+GGC-HCL High	22.5 ± 0.7	45.3 ± 1.3	64.8 ± 1.8^{bc}	85.1 ± 1.8^{bc}	105.3 ± 2.5^{bc}	129.2 ± 2.8^{c}	150.4 ± 2.3^{cd}
MZD+GGC-HCL Low	22.8 ± 0.8	43.8 ± 1.5	63.3 ± 2.4^{bc}	81.9 ± 2.1^{bc}	100.1 ± 2.3^{bc}	120.7 ± 3.7^{bc}	142.2 ± 4.4^{bcd}
MZD+GGC-La High	23.6 ± 2.1	48.1 ± 3.1	71.2 ± 4.2^{ac}	92.0 ± 5.2^{c}	112.3 ± 7.5^{c}	130.2 ± 8.7^{c}	151.2 ± 9.4^{cd}
MZD+GGC-La Low	22.5 ± 1.0	45.7 ± 1.4	60.3 ± 5.9^{b}	87.5 ± 2.8^{bc}	111.1 ± 2.7^{c}	134.1 ± 3.1^{c}	158.6 ± 3.8^{d}
MZD+GSH High	22.1 ± 0.7	43.9 ± 1.5	63.1 ± 1.7^{bc}	83.1 ± 2.6^{bc}	102.2 ± 3.3^{bc}	125.1 ± 4.5^{bc}	148.9 ± 5.2^{cd}
MZD+GSH Low	23.6 ± 1.1	43.7 ± 2.9	64.3 ± 3.2^{bc}	85.4 ± 4.3^{bc}	105.2 ± 4.7^{bc}	125.1 ± 4.6^{bc}	150.1 ± 4.9^{cd}
MZD	23.8 ± 1.1	44.9 ± 2.3	68.1 ± 3.1^{bc}	84.0 ± 3.5^{bc}	103.5 ± 3.4^{bc}	124.1 ± 3.3^{bc}	144.1 ± 4.4^{bcd}
ANOVA P-value	0.7734	0.1417	0.0250	0.0021	0.0003	<0.0001	<0.0001

Values are presented as mean ± SEM. Within a column, values not sharing a common superscript letter (a, b, c, d) are significantly different ($p < 0.05$).

Table 2.4: 3-Day food intake (grams).

	3-Day Food Intake (g)						
Diet	**Day 3**	**Day 6**	**Day 9**	**Day 12**	**Day 15**	**Day 18**	**Day 21**
ZnA Control	43.4 ± 1.0^{ac}	51.5 ± 1.5^{a}	56.8 ± 2.2	58.8 ± 2.3	62.7 ± 2.2^{a}	66.3 ± 2.5^{a}	67.9 ± 2.5^{a}
MZD+GGC-Bis High	42.5 ± 1.1^{a}	45.2 ± 1.4^{b}	48.4 ± 1.6	51.0 ± 2.0	51.0 ± 2.3^{b}	53.9 ± 1.5^{b}	53.4 ± 2.6^{bd}
MZD+GGC-Bis Low	42.7 ± 0.8^{a}	46.1 ± 1.3^{bc}	50.1 ± 1.7	54.9 ± 1.9	53.7 ± 3.4^{bc}	55.6 ± 3.4^{b}	50.8 ± 3.4^{b}
MZD+GGC-HCL High	46.0 ± 1.1^{bc}	49.4 ± 1.5^{ac}	51.0 ± 1.1	57.5 ± 1.7	58.5 ± 1.8^{ac}	64.5 ± 1.7^{ac}	63.5 ± 0.9^{ac}
MZD+GGC-HCL Low	46.9 ± 1.0^{b}	49.4 ± 1.6^{ac}	50.4 ± 1.6	54.6 ± 2.2	55.2 ± 2.8^{bc}	59.7 ± 2.4^{bc}	62.1 ± 1.9^{ac}
MZD+GGC-La High	41.3 ± 1.0^{a}	46.4 ± 1.1^{bc}	51.2 ± 1.2	53.7 ± 1.4	54.6 ± 2.2^{bc}	55.5 ± 1.4^{b}	58.4 ± 1.3^{cd}
MZD+GGC-La Low	41.8 ± 0.7^{a}	47.5 ± 1.6^{bc}	49.8 ± 2.2	54.2 ± 2.1	59.0 ± 2.6^{ac}	62.0 ± 2.1^{acd}	63.3 ± 2.2^{ac}
MZD+GSH High	42.1 ± 1.2^{a}	46.2 ± 1.0^{bc}	49.6 ± 2.1	54.4 ± 2.2	55.7 ± 1.8^{bc}	58.5 ± 1.9^{bcd}	61.5 ± 2.7^{ac}
MZD+GSH Low	41.9 ± 0.9^{a}	44.8 ± 1.3^{b}	49.1 ± 1.6	52.5 ± 1.5	54.0 ± 1.1^{bc}	56.7 ± 1.7^{bd}	60.3 ± 2.1^{c}
MZD	42.5 ± 1.1^{a}	46.3 ± 1.4^{bc}	50.9 ± 1.2	51.8 ± 1.9	54.8 ± 0.8^{bc}	56.0 ± 2.4^{bd}	57.1 ± 2.3^{bc}
ANOVA P-value	0.0009	0.0145	0.0706	0.1561	0.0305	0.0006	<0.0001

Values are presented as mean ± SEM. Within a column, values not sharing a common superscript letter (a, b, c, d) are significantly different ($p < 0.05$).

Table 2.5: Food efficiency ratio (3-Day Body weight gain / 3-Day Food Intake).

	Food Efficiency Ratio						
Diet	**Day 3**	**Day 6**	**Day 9**	**Day 12**	**Day 15**	**Day 18**	**Day 21**
ZnA Control	0.57 ± 0.03	0.53 ± 0.02^{a}	0.45 ± 0.03	0.42 ± 0.03	0.40 ± 0.01^{a}	0.40 ± 0.03	0.41 ± 0.01^{a}
MZD+GGC-Bis High	0.56 ± 0.01	0.42 ± 0.03^{b}	0.37 ± 0.02	0.34 ± 0.02	0.30 ± 0.02^{bc}	0.35 ± 0.04	0.28 ± 0.03^{bc}
MZD+GGC-Bis Low	0.58 ± 0.02	0.46 ± 0.02^{bcd}	0.38 ± 0.02	0.35 ± 0.03	0.30 ± 0.03^{bc}	0.32 ± 0.02	0.26 ± 0.04^{b}
MZD+GGC-HCL High	0.51 ± 0.01	0.48 ± 0.02^{abcd}	0.40 ± 0.02	0.36 ± 0.02	0.36 ± 0.02^{ac}	0.38 ± 0.02	0.35 ± 0.02^{ac}
MZD+GGC-HCL Low	0.51 ± 0.02	0.45 ± 0.02^{bc}	0.41 ± 0.03	0.36 ± 0.02	0.34 ± 0.02^{ac}	0.36 ± 0.02	0.36 ± 0.03^{a}
MZD+GGC-La High	0.56 ± 0.04	0.51 ± 0.02^{ad}	0.44 ± 0.02	0.37 ± 0.02	0.34 ± 0.04^{ac}	0.31 ± 0.01	0.35 ± 0.03^{ac}
MZD+GGC-La Low	0.54 ± 0.01	0.49 ± 0.02^{acd}	0.29 ± 0.12	0.50 ± 0.11	0.40 ± 0.02^{a}	0.38 ± 0.02	0.39 ± 0.02^{a}
MZD+GSH High	0.53 ± 0.02	0.47 ± 0.02^{abcd}	0.39 ± 0.03	0.37 ± 0.01	0.34 ± 0.02^{ac}	0.39 ± 0.02	0.39 ± 0.02^{a}
MZD+GSH Low	0.56 ± 0.02	0.44 ± 0.03^{bc}	0.42 ± 0.03	0.40 ± 0.02	0.37 ± 0.02^{ac}	0.35 ± 0.03	0.41 ± 0.03^{a}
MZD	0.57 ± 0.02	0.45 ± 0.03^{bcd}	0.46 ± 0.02	0.31 ± 0.03	0.36 ± 0.03^{ac}	0.38 ± 0.03	0.35 ± 0.02^{ac}
ANOVA P-value	0.2356	0.0187	0.3107	0.1232	0.0493	0.1865	0.0006

Values are presented as mean ± SEM. Within a column, values not sharing a common superscript letter (a, b, c, d) are significantly different ($p < 0.05$).

Table 2.6: Tissue weights (grams) at Day 21.

Diet	**Liver**	**Kidney**	**Testes**	**Heart**	**Spleen**
ZnA Control	$11.66 \pm 0.93^{a,e}$	2.24 ± 0.10^{a}	2.57 ± 0.05	0.93 ± 0.05	0.75 ± 0.04^{a}
MZD+GGC-Bis High	$9.00 \pm 0.39^{b,c}$	1.79 ± 0.08^{b}	2.57 ± 0.11	0.86 ± 0.04	$0.55 \pm 0.03^{b,c}$
MZD+GGC-Bis Low	$9.37 \pm 0.52^{b,c,d}$	$1.84 \pm 0.07^{b,c}$	2.56 ± 0.04	0.87 ± 0.05	0.54 ± 0.03^{b}
MZD+GGC-HCL High	$10.79 \pm 0.32^{a,d}$	$2.01 \pm 0.04^{c,d}$	2.74 ± 0.13	0.91 ± 0.03	$0.63 \pm 0.02^{b,d}$
MZD+GGC-HCL Low	10.86 ± 0.44^{a}	$2.05 \pm 0.09^{a,d}$	2.63 ± 0.08	0.90 ± 0.03	$0.67 \pm 0.04^{a,d}$
MZD+GGC-La High	$10.67 \pm 0.40^{a,d}$	$1.98 \pm 0.04^{b,d}$	2.64 ± 0.07	0.85 ± 0.02	$0.63 \pm 0.04^{b,d}$
MZD+GGC-La Low	12.30 ± 0.67^{e}	$2.09 \pm 0.07^{a,d}$	2.52 ± 0.06	0.92 ± 0.03	$0.65 \pm 0.03^{c,d}$
MZD+GSH High	$10.62 \pm 0.42^{a,d}$	$2.00 \pm 0.09^{c,d}$	2.61 ± 0.10	0.90 ± 0.04	$0.65 \pm 0.06^{a,d,e}$
MZD+GSH Low	$10.66 \pm 0.29^{a,d}$	$1.93 \pm 0.03^{b,d}$	2.56 ± 0.05	0.88 ± 0.03	$0.56 \pm 0.02^{b,c,e}$
MZD	$10.25 \pm 0.37^{a,c}$	$1.99 \pm 0.08^{b,d}$	2.66 ± 0.09	0.87 ± 0.02	$0.57 \pm 0.03^{b,c,e}$
ANOVA P-Value	0.0008	0.0037	0.8256	0.8542	0.0007

* Values are presented as mean ± SEM.

[a,b,c,d,e] Within a column, values not sharing a common superscript letter are significantly different ($p < 0.05$).

Table 2.7: Relative tissue weight (Tissue g / Body Weight g) at Day 21.

Diet	**Liver**	**Kidney**	**Testes**	**Heart**	**Spleen**
ZnA Control	0.0417 ± 0.0029^{a}	0.0080 ± 0.0001	0.0093 ± 0.0004^{a}	0.0033 ± 0.0002	0.0027 ± 0.0001
MZD+GGC-Bis High	0.0394 ± 0.0006^{a}	0.0079 ± 0.0002	0.0113 ± 0.0004^{b}	0.0038 ± 0.0001	0.0024 ± 0.0001
MZD+GGC-Bis Low	0.0398 ± 0.0014^{a}	0.0079 ± 0.0002	$0.0110 \pm 0.0004^{b,c}$	0.0037 ± 0.0001	0.0023 ± 0.0001
MZD+GGC-HCL High	$0.0416 \pm 0.0009^{a,c}$	0.0078 ± 0.0002	$0.0106 \pm 0.0005^{b,c,d}$	0.0035 ± 0.0001	0.0024 ± 0.0001
MZD+GGC-HCL Low	$0.0427 \pm 0.0008^{a,c}$	0.0081 ± 0.0002	$0.0104 \pm 0.0003^{b,c,d}$	0.0035 ± 0.0001	0.0026 ± 0.0001
MZD+GGC-La High	$0.0435 \pm 0.0012^{b,c}$	0.0081 ± 0.0002	$0.0108 \pm 0.0003^{b,c}$	0.0035 ± 0.0001	0.0025 ± 0.0001
MZD+GGC-La Low	0.0467 ± 0.0007^{b}	0.0080 ± 0.0002	$0.0097 \pm 0.0003^{a,d}$	0.0035 ± 0.0001	0.0025 ± 0.0001
MZD+GSH High	$0.0422 \pm 0.0008^{a,c}$	0.0080 ± 0.0002	$0.0104 \pm 0.0004^{b,c,d}$	0.0036 ± 0.0001	0.0026 ± 0.0002
MZD+GSH Low	$0.0424 \pm 0.0007^{a,c}$	0.0077 ± 0.0002	$0.0103 \pm 0.0003^{a,c}$	0.0035	0.0023 ± 0.0001
MZD	$0.0414 \pm 0.0009^{a,c}$	0.0080 ± 0.0002	$0.0107 \pm 0.0002^{c,d}$	0.0035 ± 0.0001	0.0023 ± 0.0001
ANOVA P-Value	0.0097	0.9258	0.0071	0.1343	0.1294

* Values are presented as mean ± SEM.

[a,b,c,d] Within a column, values not sharing a common superscript letter are significantly different ($p < 0.05$).

Table 2.8: Liver and Erythrocyte Glutathione Concentrations (µM).

Diet	Total Glutathione	GSSG	GSH	GSH:GSSG Ratio
Liver				
ZnA Control	$4.96 \pm 0.33^{a,b,c,d}$	0.80 ± 0.04^{a}	$4.15 \pm 0.32^{a,b}$	5.24 ± 0.44
MZD+GGC-Bis High	$5.37 \pm 0.26^{a,b,c,d,e}$	0.74 ± 0.05^{a}	$4.64 \pm 0.24^{a,b,c}$	6.45 ± 0.42
MZD+GGC-Bis Low	$5.42 \pm 0.17^{a,b,e}$	0.76 ± 0.03^{a}	$4.66 \pm 0.19^{a,c}$	6.27 ± 0.42
MZD+GGC-HCL High	5.86 ± 0.17^{e}	0.86 ± 0.04^{a}	5.00 ± 0.16^{c}	5.93 ± 0.29
MZD+GGC-HCL Low	5.91 ± 0.20^{e}	0.85 ± 0.04^{a}	5.06 ± 0.19^{c}	6.06 ± 0.33
MZD+GGC-La High	$4.53 \pm 0.50^{c,d}$	0.58 ± 0.09^{b}	$3.95 \pm 0.43^{a,b}$	7.73 ± 1.17
MZD+GGC-La Low	$5.18 \pm 0.35^{a,b,c,d,e}$	0.77 ± 0.05^{a}	$4.41 \pm 0.31^{a,b,c}$	5.71 ± 0.20
MZD+GSH High	$5.50 \pm 0.26^{a,c,e}$	0.83 ± 0.04^{a}	$4.66 \pm 0.24^{a,c}$	5.67 ± 0.32
MZD+GSH Low	$4.64 \pm 0.28^{b,d}$	0.78 ± 0.06^{a}	3.87 ± 0.26^{b}	5.17 ± 0.41
MZD	$5.13 \pm 0.36^{a,b,c,d,e}$	0.73 ± 0.07^{a}	$4.40 \pm 0.31^{a,b,c}$	6.41 ± 0.62
ANOVA P-Value	0.0230	0.0170	0.0327	0.0589
Erythrocyte				
ZnA Control	0.68 ± 0.04	0.08 ± 0.02	0.60 ± 0.03^{a}	10.47 ± 1.95
MZD+GGC-Bis High	0.63 ± 0.03	0.04 ± 0.01	0.59 ± 0.02^{a}	24.03 ± 6.06
MZD+GGC-Bis Low	0.63 ± 0.03	0.05 ± 0.01	0.57 ± 0.03^{a}	18.56 ± 5.12
MZD+GGC-HCL High	0.69 ± 0.05	0.06 ± 0.01	$0.64 \pm 0.04^{a,b}$	11.61 ± 1.23
MZD+GGC-HCL Low	0.66 ± 0.03	0.07 ± 0.01	0.59 ± 0.03^{a}	10.03 ± 1.41
MZD+GGC-La High	0.63 ± 0.06	0.07 ± 0.01	0.57 ± 0.06^{a}	8.09 ± 1.08
MZD+GGC-La Low	0.80 ± 0.05	0.07 ± 0.01	0.73 ± 0.04^{b}	11.13 ± 0.86
MZD+GSH High	0.64 ± 0.04	0.05 ± 0.01	0.59 ± 0.03^{a}	15.44 ± 3.55
MZD+GSH Low	0.70 ± 0.04	0.05 ± 0.01	$0.65 \pm 0.03^{a,b}$	20.71 ± 7.49
MZD	0.66 ± 0.03	0.05 ± 0.01	0.62 ± 0.03^{a}	15.71 ± 3.61
ANOVA P-Value	0.0812	0.0947	0.0386	0.0928

[*] Values are presented as mean ± SEM.

[a,b,c,d,e] Within a column and tissue type, values not sharing a common superscript letter are significantly different ($p < 0.05$).

Table 2.9: Plasma TBARS concentrations, expressed as malondialdehyde (MDA) equivalents.

Diet	**MDA (uM)**
ZnA Control	0.935 ± 0.046
MZD+GGC-Bis High	0.888 ± 0.040
MZD+GGC-Bis Low	0.915 ± 0.046
MZD+GGC-HCL High	0.911 ± 0.038
MZD+GGC-HCL Low	0.861 ± 0.026
MZD+GGC-La High	0.831 ± 0.038
MZD+GGC-La Low	0.825 ± 0.034
MZD+GSH High	0.861 ± 0.035
MZD+GSH Low	0.817 ± 0.030
MZD	0.896 ± 0.064
ANOVA P-Value	0.4402

* Values are presented as mean ± SEM. There were no significant differences among groups.

Table 2.10: Erythrocyte Hemolysis Assay (% hemolysis over time).

	% Hemolysis								
Diet	**0 Min**	**7.5 Min**	**15 Min**	**22.5 Min**	**30 Min**	**37.5 Min**	**45 Min**	**52.5 Min**	**60 Min**
ZnA Control	4.4 ± 0.9	5.6 ± 1.4	7.1 ± 1.1	12.7 ± 3.2	$33.5 \pm 9.2^{*}$	64.3 ± 14.6	81.8 ± 5.2	92.5 ± 6.7	98.6 ± 8.1
MZD+GGC-Bis High	4.2 ± 1.2	8.9 ± 4.1	17.3 ± 6.6	34.0 ± 9.7	55.0 ± 11.1	84.9 ± 8.1	95.5 ± 3.7	92.6 ± 4.8	99.3 ± 6.3
MZD+GGC-Bis Low	5.2 ± 1.1	4.4 ± 1.5	5.2 ± 1.5	19.5 ± 6.3	62.9 ± 11.8	79.2 ± 2.5	97.1 ± 3.7	104.3 ± 3.7	101.5 ± 4.9
MZD+GGC-HCL High	3.7 ± 1.3	4.5 ± 1.4	6.5 ± 2.4	13.5 ± 5.2	40.7 ± 10.3	78.8 ± 13.1	88.4 ± 4.9	93.1 ± 4.7	103.6 ± 5.2
MZD+GGC-HCL Low	3.3 ± 1.3	2.7 ± 1.3	10.2 ± 4.4	11.8 ± 5.9	32.9 ± 10.6	68.4 ± 13.9	91.3 ± 4.0	91.7 ± 3.1	94.1 ± 3.3
MZD+GGC-La High	4.4 ± 1.4	5.0 ± 1.4	8.0 ± 2.6	27.1 ± 8.9	48.9 ± 12.3	55.8 ± 18.4	85.0 ± 6.8	82.1 ± 3.7	96.2 ± 6.8
MZD+GGC-La Low	3.2 ± 1.2	5.8 ± 1.3	12.2 ± 7.4	21.0 ± 9.9	34.2 ± 12.1	51.8 ± 8.2	77.7 ± 6.2	82.4 ± 9.0	91.4 ± 5.7
MZD+GSH High	6.1 ± 1.5	4.1 ± 1.4	5.0 ± 1.9	10.8 ± 3.7	36.3 ± 10.3	58.1 ± 21.5	80.4 ± 10.7	89.4 ± 13.5	112.9 ± 6.4
MZD+GSH Low	3.6 ± 1.1	4.3 ± 1.4	3.8 ± 2.5	15.5 ± 4.5	45.0 ± 11.6	77.0 ± 7.3	90.2 ± 4.2	90.1 ± 2.5	104.8 ± 8.7
MZD	3.8 ± 1.6	4.1 ± 1.2	9.2 ± 3.2	25.6 ± 11.0	$65.2 \pm 8.4^{*}$	84.4 ± 5.5	93.1 ± 4.1	94.4 ± 4.1	101.9 ± 3.0
ANOVA P-value	0.8605	0.6455	0.4044	0.3434	0.2671	0.5189	0.2224	0.5410	0.4100

Values are presented as mean ± SEM.

*When comparing only the ZnA Control and MZD groups, a significant difference was observed at this time point ($p < 0.05$).

Bibliography

[1] Salem A. Habib et al. "Pro-inflammatory adipocytokines, oxidative stress, insulin, Zn and Cu: interrelations with obesity in egyptian non-diabetic obese children and adolescents". en. In: *Advances in Medical Sciences* 60.2 (Sept. 2015), pp. 179–185. DOI: 10.1016/j.advms.2015.02.002.

[2] Rajagopal V. Sekhar et al. "Glutathione Synthesis Is Diminished in Patients With Uncontrolled Diabetes and Restored by Dietary Supplementation With Cysteine and Glycine". In: *Diabetes Care* 34.1 (Oct. 2010), pp. 162–167. DOI: 10.2337/dc10-1006.

[3] John P. Richie et al. "Blood Iron, Glutathione, and Micronutrient Levels and the Risk of Oral Cancer". In: *Nutrition and Cancer* 60.4 (June 2008). Publisher: Routledge _eprint: https://doi.org/10.1080/01635580801956477, pp. 474–482. DOI: 10.1080/01635580801956477.

[4] Eun Young Park et al. "Increase in the Protein-Bound Form of Glutathione in Human Blood after the Oral Administration of Glutathione". In: *Journal of Agricultural and Food Chemistry* 62.26 (July 2014). Publisher: American Chemical Society, pp. 6183–6189. DOI: 10.1021/jf501338z.

[5] Jason Allen and Ryan D. Bradley. "Effects of Oral Glutathione Supplementation on Systemic Oxidative Stress Biomarkers in Human Volunteers". In: *The Journal of Alternative and Complementary Medicine* 17.9 (Sept. 2011). Publisher: Mary Ann Liebert, Inc., publishers, pp. 827–833. DOI: 10.1089/acm.2010.0716.

[6] John P. Richie et al. "Randomized controlled trial of oral glutathione supplementation on body stores of glutathione". en. In: *European Journal of Nutrition* 54.2 (Mar. 2015), pp. 251–263. DOI: 10.1007/s00394-014-0706-z.

[7] Hoda Moazzen et al. "N-Acetylcysteine prevents congenital heart defects induced by pregestational diabetes". en. In: *Cardiovascular Diabetology* 13.1 (Feb. 2014), p. 46. DOI: 10.1186/1475-2840-13-46.

[8] Emily Ho, Gouman Chen, and Tammy M. Bray. "Supplementation of N-acetylcysteine inhibits NF B activation and protects against alloxan-induced diabetes in CD-1 mice". en. In: *The FASEB Journal* 13.13 (1999). _eprint: https://faseb.onlinelibrary.wiley.com/doi/pdf/10.1096/fasebj.13.13.1845, pp. 1845–1854. DOI: 10.1096/fasebj.13.13.1845.

[9] Lynn A Hanna et al. "The influence of gestational zinc deficiency on the fetal insulin-like growth factor axis in the rat". en. In: *Experimental Biology and Medicine* 235.2 (Feb. 2010), pp. 206–214. DOI: 10.1258/ebm.2009.009018.

[10] Yo Omata et al. "Decreased Zinc Availability Affects Glutathione Metabolism in Neuronal Cells and in the Developing Brain". In: *Toxicological Sciences* 133.1 (May 2013), pp. 90–100. DOI: 10.1093/toxsci/kft022.

[11] Qin Yan Zhu et al. "Influence of Cocoa Flavanols and Procyanidins on Free Radical-induced Human Erythrocyte Hemolysis". en. In: *Journal of Immunology Research* 12.1 (2005). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1080/17402520512331329514, p. 491658. DOI: 10.1080/17402520512331329514.

[12] John A. Polagruto et al. “Platelet Reactivity in Male Smokers Following the Acute Consumption of a Flavanol-Rich Grapeseed Extract”. In: *Journal of Medicinal Food* 10.4 (Dec. 2007). Publisher: Mary Ann Liebert, Inc., publishers, pp. 725–730. DOI: 10.1089/jmf.2007.402.

[13] Patricia I. Oteiza. “Zinc and the modulation of redox homeostasis”. en. In: *Free Radical Biology & Medicine* 53.9 (Nov. 2012), pp. 1748–1759. DOI: 10.1016/j.freeradbiomed.2012.08.568.

[14] Janet Y. Uriu-Adams and Carl L. Keen. “Zinc and reproduction: effects of zinc deficiency on prenatal and early postnatal development”. en. In: *Birth Defects Research Part B-developmental and Reproductive Toxicology* 89.4 (Aug. 2010), pp. 313–325. DOI: 10.1002/bdrb.20264.

[15] Wei Zhong et al. “Dietary Zinc Deficiency Exaggerates Ethanol-Induced Liver Injury in Mice: Involvement of Intrahepatic and Extrahepatic Factors”. en. In: *PLOS ONE* 8.10 (Oct. 2013). Publisher: Public Library of Science, e76522. DOI: 10.1371/journal.pone.0076522.

[16] Sheri Zidenberg-Cherr et al. “Ethanol-induced changes in hepatic free radical defense mechanisms and fatty-acid composition in the miniature pig”. en-US. In: *Hepatology* 13.6 (June 1991), p. 1185. DOI: 10.1002/hep.1840130627.

[17] Betty J. Mills, Robert D. Lindeman, and Calvin A. Lang. “Effect of Zinc Deficiency on Blood Glutathione Levels”. In: *The Journal of Nutrition* 111.6 (June 1981), pp. 1098–1102. DOI: 10.1093/jn/111.6.1098.

[18] Betty J. Mills, Robert D. Lindeman, and Calvin A. Lang. “Differences in Blood Glutathione Levels of Tumor-Implanted or Zinc-Deficient Rats”. In: *The Journal of Nutrition* 111.9 (Sept. 1981), pp. 1586–1592. DOI: 10.1093/jn/111.9.1586.

[19] Qin Yan Zhu et al. “Inhibitory Effects of Cocoa Flavanols and Procyanidin Oligomers on Free Radical-Induced Erythrocyte Hemolysis”. EN. In: *Experimental Biology and Medicine* 227.5 (May 2002). Publisher: SAGE Publications, pp. 321–329. DOI: 10.1177/153537020222700504.

[20] E Niki et al. “Membrane damage due to lipid oxidation”. In: *The American Journal of Clinical Nutrition* 53.1 (Jan. 1991), 201S–205S. DOI: 10.1093/ajcn/53.1.201S.

[21] Jun Li et al. “Dietary supplementation with cysteine prodrugs selectively restores tissue glutathione levels and redox status in protein-malnourished mice1”. In: *The Journal of Nutritional Biochemistry* 13.10 (Oct. 2002), pp. 625–633. DOI: 10.1016/S0955-2863(02)00218-8.

[22] Boyd L. O’Dell, Jimmy D. Browning, and Philip G. Reeves. “Zinc Deficiency Increases the Osmotic Fragility of Rat Erythrocytes”. In: *The Journal of Nutrition* 117.11 (Nov. 1987), pp. 1883–1889. DOI: 10.1093/jn/117.11.1883.

Chapter 3

Maternal Biochemical Profiles Across Prenatal Alcohol Exposure Trajectories: The Modifying Action of Multivitamin/Multimineral Supplementation.

3.1 Introduction

3.1.1 Prevalence

FASD is a leading preventable cause of global neurodevelopmental disability. Prevalence estimates vary by region and study design. Systematic reviews report FAS affects approximately 15 per 10,000 people, and FASD affects roughly 7.7 per 1,000 births[1,2]. Examinations of high-risk populations show rates 10 to 40 times higher than in the general population[3]. A rural South African study found prevalence up to 31%[4]. Clinical reassessment studies report significant misdiagnosis rates, hindering early intervention[5,6]. The public health burden

of FASD includes increased use of outpatient and emergency services and greater expenditures in social, educational, and judicial systems[7,8]. This burden demonstrates the need for prevention strategies and early diagnostic measures.

3.1.2 Oxidative Stress

Experimental models demonstrated PAE disrupts the body's reduction/oxidation (redox) balance and impairs nutrient homeostasis. The oxidative metabolism of ethanol by enzymes such as ADH and CYP2E1 generates an overproduction of ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals[9,10]. Excess ROS induce oxidative stress, causing damage to lipids, proteins, and DNA in maternal and fetal tissues[11,12]. Ethanol metabolism also depletes critical antioxidants, such as GSH, and compromises the activity of defense enzymes like SOD[12,13]. This redox imbalance is compounded by ethanol's interference with the absorption and utilization of critical micronutrients. It impairs zinc homeostasis[14–20], alters copper status[21–25], and disrupts iron metabolism[26–29]. These nutrient-specific disruptions compromise oxidative defense systems. The combination of oxidative stress and nutrient-mediated effects contributes to FASD manifestation by altering cell signaling and impairing organogenesis[12,13].

The fetal system's immaturity magnifies its susceptibility to ethanol-induced oxidative injury. Fetal CYP2E1 activity, for example, is substantially lower than adult levels, approximately 40% by the second trimester[30]. This reduced capacity to metabolize ethanol prolongs fetal exposure to ethanol and its metabolites. The developing brain is especially susceptible to such oxidative damage. Its high oxygen consumption rate, abundant peroxidizable unsaturated fatty acids, and comparatively low antioxidant reserves make it particularly vulnerable to the peroxidation cascades PAE-induced ROS initiate[12,31].

3.1.3 Nutrition Depletion

Zinc is necessary for cellular growth, differentiation, and central nervous system development, supporting processes such as nucleic acid metabolism and cell signaling[14]. Alcohol consumption during pregnancy can trigger an APR, prompting maternal hepatic MT synthesis, a protein sequestering zinc in the liver[14]. This sequestration reduces maternal plasma zinc available for placental transport. Lower maternal and fetal zinc levels have been observed in pregnancies involving alcohol use[16]. Fetal zinc deficiency is associated with increased risk for developmental abnormalities[14,16].

Copper is a necessary cofactor for many enzymes active in energy metabolism, oxidative injury protection, vascular development, and nervous system formation[24,32]. Alcohol consumption during pregnancy can lower maternal copper concentrations[23,25]. Increased biliary copper excretion in response to alcohol exposure contributes to this reduction[23]. Compromised maternal copper status can disrupt fetal vascular development and reduce copper dependent antioxidant enzyme activity, such as CuZnSOD. These disturbances are linked to abnormal central nervous system formation and fetal structural defects[24].

PAE affects iron metabolism, a nutrient important for fetal growth and neurodevelopment. Heavy alcohol intake can suppress hepatic hepcidin transcription. Hepcidin suppression elevates intestinal iron transporter protein expression and increases maternal iron absorption[33]. Maternal iron dysregulation can elevate maternal liver oxidative stress and modify fetal iron availability. PAE is linked to a higher incidence of infant iron deficiency, particularly following maternal binge drinking[28]. The development of infant iron deficiency under these conditions, where maternal iron absorption may be elevated, is mediated by an APR, which increases ferritin and decreases serum iron. This reduction in circulating iron limits fetal availability[27,28].

3.1.4 Nutrient Based Mitigation

Animal studies indicate maternal nutritional interventions may counter some adverse PAE effects. Rodent studies demonstrated antioxidant supplementation reduced ethanol-initiated oxidative stress. Pretreatment with vitamin E, or antioxidants like vitamin C, diminished lipid peroxidation markers and oxidative DNA damage in fetal brain tissue. These interventions lessened apoptosis and supported neuronal survival[13,34]. Prenatal N-acetylcysteine, a glutathione precursor, decreased ROS generation and rebalanced the intracellular redox state in ethanol-exposed embryos[31].

Multivitamin interventions with antioxidant micronutrients showed benefits in animal models. Prenatal multivitamin and mineral supplementation, designed to restore antioxidant levels and critical trace elements, improved fetal growth and lessened ethanol-induced mineral homeostasis disruptions[14,32]. Gestational iron supplementation in ethanol-exposed rodents reversed fetal anemia and partially normalized brain weight deficits[29].

Zebrafish models offered additional evidence. Supplementation with retinoic acid, an active vitamin A metabolite, rescued ethanol induced morphological defects during gastrulation and somitogenesis. This finding indicates nutrient based strategies may correct disrupted developmental signaling[35].

3.1.5 Alcohol Trajectories

Many PAE studies used dichotomous measures, classifying women as "drinkers" or "non-drinkers." These classifications omit detailed variations in alcohol consumption quantity, frequency, and timing. These details are important for understanding the biological impact of exposure and for investigating dose response relationships, which may be non-linear[36].

Alcohol trajectory modeling offers a more detailed method, grouping women into distinct longitudinal consumption patterns[37]. These techniques retain specific alcohol intake information across gestational periods. This approach reduces misclassification common in dichotomous categorizations. Researchers can then examine the developmental course of

drinking behavior across relevant timeframes. The resulting trajectory groups improve comparability across studies and may reveal insights into PAE effects not apparent from binary classification[36–38].

3.1.6 Knowledge Gaps and Study Rationale

Maternal alcohol consumption disrupts maternal redox balance and nutrient homeostasis, processes affecting healthy fetal neurodevelopment. Prior investigations documented ethanol's role in generating ROS and diminishing levels of key antioxidants and trace elements. Experimental evidence from animal models linked these physiological insults to morphological and neurobehavioral fetal deficits and suggest that nutrient-based interventions might counteract some adverse effects.

A knowledge gap persists. Direct human evidence lacks clarification on how MVM supplementation, diverse maternal alcohol consumption patterns, and maternal biochemical markers of oxidative stress, antioxidant defense, and nutrient status interact during pregnancy to impact fetal outcomes. Current human research seldom integrates longitudinal exposure data with the evaluation of nutritional interventions and their impacts on fetal development.

This study addresses this deficit. Growth mixture modeling identifies distinct maternal alcohol intake trajectories throughout gestation. This allows for an examination of whether MVM supplementation during pregnancy associates with improved maternal biochemical profiles, including antioxidant defense activities and micronutrient levels, across alcohol exposure groups. Furthermore, this study investigates the effects of MVM supplementation on infant growth characteristics and neurodevelopmental outcomes in relation to maternal alcohol consumption patterns. This investigation clarifies the interplay between prenatal alcohol exposure dynamics, nutritional support, maternal physiological responses, and infant development within a human cohort.

3.2 Materials and Methods

3.2.1 Study Design and Participants

Data originated from a prospective cohort study within the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD), funded by the National Institute on Alcohol Abuse and Alcoholism. The parent study occurred from April 2008 to August 2012 at two prenatal care facilities in Western Ukraine: the Rivne Regional Medical Diagnostic Center and the Khmelnytsky Perinatal Center. These sites participated in OMNI-Net, a network for birth defects prevention. CIFASD aimed to characterize FASD, develop improved diagnostic and prevention strategies, and investigate maternal nutrition as a modifying factor. Details of the methods and scope of CIFASD studies have been described elsewhere[39,40]. Institutional review boards at the University of California San Diego and Lviv Medical University in Ukraine approved the study protocol. All participants gave written informed consent before enrollment.

Pregnant women attending routine prenatal visits were screened for alcohol consumption. Women entered an alcohol exposed (AE) group if reporting consumption, during the month near conception or the most recent pregnancy month, meeting one of the following criteria: weekly binge-drinking episodes (four to five or more standard alcoholic drinks per occasion), five or more episodes of three to four standard drinks, or ten or more episodes of one to two standard drinks. Following recruitment of an AE participant, the next woman presenting who met criteria for low or no alcohol exposure entered a comparison group. Comparison group criteria required consuming fewer than two drinks per occasion, two or fewer drinks per week near conception, and no alcohol consumption during the most recent pregnancy month.

Participants from both exposure groups were randomly allocated to one of three intervention arms. The first arm received standard prenatal care; prenatal vitamins were recommended but not supplied by the study. The second arm received a daily MVM supplement

(Theravit®). The third arm received the daily MVM supplement and an additional 750 mg of supplemental choline. The MVM and choline supplements were supplied free of charge; compliance was measured by self-report.

Screening for Risky Drinking

The TWEAK questionnaire screened participants for risky drinking behavior[41]. Points were assigned based on participant responses: two points for *Tolerance* (consuming six or more drinks before adverse effects), two points if others expressed *Worry* about the participant's drinking, one point for needing an *Eye-opener* (morning drink), one point for *Amnesia* (memory loss after drinking), and one point for stating a desire to *Kut down* (cut down) drinking[42,43]. The maximum TWEAK score is seven points. A score of two or more points defined a positive screen for risky alcohol consumption. A positive screen determined participant eligibility for the alcohol-exposed recruitment group in the parent study.

3.2.2 Data Collection and Measures

Maternal Interviews and Visit Schedule

Data acquisition consisted of maternal interviews and biological sampling at enrollment and a follow-up visit near 32 weeks gestation. Interviews recorded demographics, pre-pregnancy body mass index (BMI), socioeconomic status (SES) (Hollingshead ratings), health behaviors like tobacco use, existing supplement use, and pregnancy characteristics. Maternal blood samples were obtained at enrollment and the third-trimester visit for biomarker analysis.

We categorized gestational timing using the method of Sowell et al. (2018)[44]. This method separated visits into early/mid pregnancy (< 26 weeks) or late pregnancy (≥ 26 weeks). If both visits for a participant occurred within the early/mid pregnancy window, we excluded the second visit to prevent data duplication.

Alcohol Consumption Assessment

Maternal alcohol consumption was quantified using a timeline follow-back (TLFB) method[45] for women reporting any lifetime drinking. This method recorded day-by-day alcohol intake (type, quantity, frequency) for a typical week near conception and for the two weeks preceding the enrollment interview. At the follow-up visit, women reported changes in alcohol consumption. If consumption changed, a TLFB for the previous seven days was completed. Alcohol quantities were converted to absolute ounces of alcohol (ozAA) per day; one standard drink equaled 0.5 ozAA. Standardized screening tools for risky drinking, like the TWEAK[41], were administered.

Variable Construction

Maternal Smoking Classification We created a composite measure from self-reported smoking status and daily consumption data. This measure encompassed individuals who reported no smoking or who provided daily smoking counts.

Maternal Supplement Use Classification Supplement names reported by participants were examined, with misspellings corrected and minor spelling variations standardized. A list of unique supplement names was then compiled, and each supplement's composition was reviewed. Supplements were categorized as single-nutrient, dual-nutrient, or multivitamin. Participant use was recategorized under these classifications. Individuals were considered MVM users if they took a multinutrient supplement, or any combination of three or more single-nutrient items, or a mixture of single-nutrient and dual-nutrient products. They were considered non-MVM users if they had only one or two single-nutrient items, a single dual-nutrient product, or did not take any nutrient supplements.

Maternal Vitamin Use Grouping Participants were assigned to one of three maternal vitamin use groups: (1) No MVM Use (No MVM), individuals reporting no or inconsistent

MVM use ($\leq$ 3 times weekly); (2) MVM Since Enrollment (MVM SE), individuals initiating consistent MVM use at enrollment; and (3) MVM Prior to Enrollment (MVM PE), individuals maintaining consistent MVM use initiated pre-enrollment. The MVM SE and MVM PE groups were further stratified by choline supplementation initiated at enrollment.

Maternal Biological Analyses

Blood Sample Collection and Plasma Preparation From each participant, a 25 mL blood sample was collected into tubes containing either heparin or ethylenediaminetetraacetic acid (EDTA) after enrollment and again at approximately 32 weeks of gestation. Each sample was centrifuged at 1500 x g for 10 minutes at 4 °C. The resulting plasma was aliquoted into tubes and frozen at −80 °C pending shipment to the United States for analysis.

Hemoglobin (Hb) Hemoglobin (Hb) concentrations were determined using a cyanmethemoglobin-based method. Red blood cell hemolysates, prepared by lysing erythrocytes with deionized water and subsequently stored at −80 °C, were thawed and diluted 1:30 (v/v) with deionized water. For the assay, 10 µL of this diluted hemolysate was combined with 190 µL of Drabkin's reagent in microplate wells. Drabkin's reagent (Sigma D5941, St. Louis, MO, USA) was prepared by reconstituting reagent powder in 1000 mL deionized water with 0.5 mL of 30 % Brij 35 solution; the reagent was stored at room temperature in a foil-covered bottle. This reagent oxidized hemoglobin to methemoglobin using alkaline potassium ferricyanide; the methemoglobin then reacted with potassium cyanide to form stable cyanmethemoglobin. After a minimum 15 min incubation at room temperature, the absorbance of cyanmethemoglobin was measured at 540 nm. Hemoglobin concentrations were quantified against a standard curve prepared from cyanmethemoglobin standards.

Erythrocyte Superoxide Dismutase (SOD) Erythrocyte SOD activity was determined spectrophotometrically through the inhibition of pyrogallol autooxidation. The assay measured SOD catalyzed dismutation of superoxide radicals, which competed with superoxide-

mediated pyrogallol autooxidation[46]. The pyrogallol autooxidation rate was monitored by the absorbance increase at 420 nm in a basic buffer. One unit of SOD activity represented the amount of enzyme in the hemolysate that inhibited the pyrogallol autooxidation rate by 50%. SOD activity was expressed as Units/g Hb.

Erythrocyte Glutathione Peroxidase (GPx) Erythrocyte GPx activity was quantified in red blood cell hemolysates. The assay employed a coupled enzymatic reaction[47,48]. This reaction linked the GPx-catalyzed reduction of a hydroperoxide substrate, with GSH as the electron donor, to nicotinamide adenine dinucleotide phosphate (NADPH) oxidation by exogenous glutathione reductase (GRed). The decrease in NADPH concentration was monitored spectrophotometrically by the absorbance change at 340 nm over time. GPx activity was expressed as µmol NADPH ox/min/g Hb.

Erythrocyte Glutathione Reductase (GRed) GRed activity was determined using a kinetic spectrophotometric assay adapted from established protocols[49,50]. The assay quantified the NADPH oxidation rate by monitoring absorbance decrease at 340 nm. GRed catalyzed this reaction, utilizing NADPH as an electron donor to reduce GSSG. Erythrocyte hemolysates were combined with a reaction buffer containing GSSG, and NADPH addition initiated the reaction. Enzyme activity was calculated from the absorbance change of NADPH. GRed activity was expressed as µmol NADPH ox/min/g Hb.

Plasma Thiobarbituric Acid Reactive Substances (TBARS) Lipid peroxidation was assessed by quantifying plasma TBARS. The assay involved reacting MDA and other aldehydic lipid peroxidation products with TBA. This reaction, performed under acidic conditions and heat, yields a fluorescent adduct[51]. Upon blood collection, plasma samples were treated with butylated hydroxytoluene (BHT) to prevent in vitro oxidation. On the day of the assay, the reaction mixture contained 3% sodium dodecyl sulfate and 10% phosphotungstic acid, which precipitated interfering macromolecules[52,53]. The fluorescent TBA

MDA adduct was extracted into 1-butanol. Its intensity was measured fluorometrically at an excitation wavelength of 515 nm and an emission wavelength of 555 nm. TBARS concentrations were determined by comparison to a standard curve generated from 1,1,3,3-tetraethoxypropane, an MDA precursor. The results are expressed as µM MDA.

Plasma Total Radical-Trapping Antioxidant Parameter (TRAP) Plasma total radical trapping antioxidant potential (TRAP) was determined by a chemiluminescence based assay[54,55]. The assay quantified the capacity of plasma antioxidants to inhibit luminol's oxidative degradation. Luminol degradation generated a chemiluminescent signal and was initiated by a constant flux of peroxyl radicals released from the thermal decomposition of 2,2'-azo-bis(2-amidinopropane) (ABAP). The TRAP value was determined from the lag phase duration before a chemiluminescence increase after adding diluted plasma to the ABAP and luminol reaction mixture. This lag phase duration was directly proportional to the total radical trapping capacity of the plasma antioxidants. Results were calibrated against a known concentration of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Data are expressed as µM Trolox equivalents.

Plasma Zinc, Iron, and Copper Plasma concentrations of zinc, iron, and copper were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Plasma aliquots were combined with Ultrex nitric acid. Incubated samples were centrifuged to pellet precipitated material. The supernatant was decanted and analyzed for mineral content using a Thermos Scientific iCAP 6300 Duo View ICP-OES Spectrometer. Data are expressed as µg mineral/mL plasma.

Plasma Ceruloplasmin (CP) Plasma CP oxidase activity was quantified by a kinetic spectrophotometric assay[56]. The assay measured the CP-catalyzed oxidation rate of o-dianisidine dihydrochloride, which produced a yellow-brown reaction product. The enzymatic reaction was stopped at two time points by adding sulfuric acid. This addition formed

a stable purple-red chromophore. The final product's absorbance was measured at 540 nm. CP activity was calculated from the absorbance change between the two time points per minute of incubation and expressed as Units/L.

Plasma High-Sensitivity C-Reactive Protein (hsCRP) Plasma high sensitivity C-reactive protein (hsCRP) concentrations were quantified with a solid-phase, chemiluminescent immunometric assay (IMMULITE®/IMMULITE® 1000 High Sensitivity CRP, Siemens Medical Solutions USA, Inc., Malvern, PA, USA) on an IMMULITE 1000 analyzer. For the assay, prediluted samples and a reagent mixture were incubated with an anti-ligand coated bead. During this incubation, sample hsCRP formed an antibody sandwich complex with bead-bound and reagent antibodies. Unbound components were removed by a centrifugal wash. A chemiluminescent substrate was added to the bead. The analyzer measured the light emission, proportional to the quantity of bound enzyme. Data are expressed as mg/L.

Plasma Ferritin Plasma ferritin concentrations were quantified using a solid-phase, two-site chemiluminescent immunometric assay (IMMULITE®/IMMULITE® 1000 Ferritin, Siemens Medical Solutions USA, Inc., Malvern, PA, USA) with an IMMULITE 1000 analyzer. This assay utilized a bead coated with monoclonal murine anti-ferritin antibody and an alkaline phosphatase-conjugated polyclonal goat anti-ferritin antibody reagent. During a 30-minute incubation, plasma ferritin formed an antibody-sandwich complex with these bead-bound and enzyme conjugated antibodies. Unbound components were removed by centrifugal washes. A chemiluminescent substrate was then introduced, and the analyzer measured the resultant signal, which was proportional to the ferritin concentration. Data are expressed as ng/mL plasma.

Plasma Soluble Transferrin Receptor (sTfR) Plasma soluble transferrin receptor (TfR) concentration measurements were performed using a commercial quantitative sandwich enzyme immunoassay (Quantikine IVD® Human sTfR Immunoassay, R&D Systems,

Inc., Minneapolis, MN, USA). In this assay, a monoclonal antibody precoated onto microplate wells bound TfR present in plasma samples or standards. After a wash step, an enzyme linked polyclonal antibody specific for TfR was added, forming an antibody-antigen-antibody sandwich. A second wash removed unbound enzyme-linked antibody. A substrate solution was then added to the wells, and color developed proportionally to the amount of TfR bound in the initial step. The reaction was terminated, and the optical density was measured spectrophotometrically. TfR concentrations were determined by comparison to a standard curve generated from known concentrations and expressed as nmol/L.

Infant Outcome Assessment

Anthropometric Measures at Birth Information on infant growth was obtained from medical records after birth. Infant outcomes included infant birthweight (grams), length (cm), head circumference (cm), and gestational age at birth (weeks).

Neurodevelopmental Assessment Infant neurodevelopment was evaluated at approximately 6 and 12 months postpartum using the BSID-II, Second Edition[57]. Trained psychologists, blinded to maternal alcohol exposure and intervention group status, administered the assessments. Primary outcomes from the BSID-II were the MDI and the Psychomotor Development Index (PDI). Both indices yielded standardized scores (population mean=100, SD=15). Scores were age-corrected for infants born before 37 weeks' gestation. The MDI assessed early cognitive and language abilities, including knowledge, problem-solving, and memory. The PDI evaluated motor skills such as body control and the coordination of large and fine muscle movements.

3.2.3 Data Pre-processing

Data Imputation

A nonparametric algorithm, `missForest`, (version 1.5)[58] in R version 4.4.2[59] based on random forests was used to address missing covariate data. Biomarker data and alcohol intake were not imputed. This algorithm accommodates mixed-type data, drawing on all available measures to improve imputation accuracy, and provides an error estimate for each imputed value.

A comprehensive dataset of covariates and analytes was used, with the imputation run for 500 trees and 100 iterations. The missing values involved marijuana use (7 missing, 1%), maternal education (3 missing, <1%), socioeconomic status (4 missing, 1%), smoking amount in pregnancy (3 missing, <1%), pre-pregnancy BMI (10 missing, 2%), pregnancy planning (5 missing, 1%), electricity availability at home (4 missing, 1%), phone availability at home (4 missing, 1%), presence of an indoor toilet (4 missing, 1%), paternal age (5 missing, 1%), total number of pregnancies (1 missing, 0%), and residential crowding (5 missing, 1%).

Mean squared errors for these measures were: marijuana use: 0.022, maternal education: 3.708, socioeconomic status: 94.79, smoking amount in pregnancy: 9.509, pre-pregnancy BMI: 11.134, pregnancy planning: 0.373, electricity availability: 0.013, phone availability: 0.162, indoor toilet: 0.134, paternal age: 24.327, total pregnancies: 1.482, residential crowding: 0.161. The low values for marijuana use, pregnancy planning, electricity availability, phone availability, indoor toilet, and residential crowding indicate good imputation performance. Higher values for socioeconomic status (94.79), paternal age (24.327), and pre-pregnancy BMI (11.134) show greater variability in their imputation. Even so, the resulting imputations remained within the original data ranges and were near the mean and median of non-missing observations.

Outlier Detection and Winsorizing

Outliers across covariates and biomarkers were visualized through Q–Q plots and histograms. Suspect values were assessed using Rosner's test, which identifies up to k candidate outliers in data assumed to be normally distributed. The test iteratively removes the most extreme values and recalculates the mean and standard deviation. Observations flagged as outliers were winsorized by substituting the next highest or lowest non-outlier value. We conducted Rosner's test in R with the `EnvStats` package (version 3.0.0)[60].

Alcohol Intake Outliers Rosner's test ($\alpha = 0.05$) was applied to daily alcohol consumption measured at conception, enrollment, and a follow-up pregnancy visit. Three significant outliers were identified at conception (6.80, 5.31, 5.15 ounces of alcohol per day). Their test statistics (R = 7.14, 5.85, 5.98) exceeded a critical threshold of approximately 3.76. One significant outlier (10.328 ounces of alcohol per day) was found at enrollment (R = 14.03). Three outliers (3.32, 2.74, 2.22 ounces of alcohol per day) appeared at follow-up (R = 5.59, 5.01, 4.31), exceeding a critical threshold near 3.56. These outliers were winsorized by substituting them with the next highest non-outlier values (4.295 for conception, 3.32 for enrollment, 1.707 for follow-up) to aid model convergence.

Biomarker Outliers Rosner's test ($\alpha = 0.05$) was also applied to second-visit data on TBARS and plasma iron. One significant outlier (4.321 µM MDA) was detected in TBARS (R = 7.175, critical threshold 3.842). One significant outlier (22.561 ng/mL) was detected for plasma iron (R = 19.314). Each was replaced with the next highest non-outlier value (2.860 µM MDA for TBARS, 3.220 ng/mL for plasma iron).

3.2.4 Statistical Analysis

Alcohol Intake Trajectory Analysis

Construction of Gestational Timeline for Alcohol Intake Three measures of daily alcohol consumption were recorded for each participant: at conception, at pregnancy recognition, and at a follow-up visit (approximately 32 weeks' gestation). Each recorded value was expressed in ounces of absolute alcohol per day. The reported consumption at conception was assigned from week 0 until the week preceding pregnancy recognition. The consumption level at pregnancy recognition was applied from that week until the follow-up visit at approximately 32 weeks' gestation. The follow-up visit measurement was applied from that visit through delivery. This process created a continuous series of weekly alcohol intake values, forming an individual consumption trajectory for each participant.

Inclusion Criteria and Data Sufficiency for Trajectory Modeling Participants with twin pregnancies were excluded from the analysis. The availability of time-point data to construct gestational drinking trajectories was reviewed. Three segments for trajectory construction were defined: conception to the week of pregnancy recognition; the week of pregnancy recognition to the follow-up visit; and the follow-up visit to birth. Each participant required data for at least two adjacent time points to form a segment. Twelve participants lacked sufficient data to establish any segments and were excluded from trajectory modeling.

Trajectory Modeling Approach and Data Aggregation Weekly measurements presented statistical modeling limitations from minimal variability and potential collinearity in later gestation. These factors compromised model convergence and accuracy. Initial attempts using only three data points (conception, recognition, follow-up) or the complete weekly record did not succeed. Alternative models, such as two-part growth models (modeling drinking probability and consumption levels separately) and censored models (treating zero consumption as below a detection threshold), were unsuitable. Both approaches en-

countered convergence issues. These convergence problems persisted when participants with no consumption were included and when they were excluded.

To manage these modeling difficulties, weekly data were aggregated into monthly averages. This aggregation reduced data dimensionality and lowered collinearity. A growth mixture model (GMM) then identified distinct alcohol consumption trajectories from the aggregated data for individuals reporting any alcohol intake. Individuals reporting no alcohol consumption were excluded from the trajectory modeling as this group formed an a priori defined subset, not a latent class.

Growth Mixture Model (GMM) Specification Mplus (version 8.11)[61] estimated a GMM to identify latent classes of similar alcohol-consumption trajectories. Two growth factors, an intercept and a slope, defined these classes. The model included seven measurement points (conception, and months 1, 2, 3, 4, 7, and 10) to reduce collinearity and stabilize estimation. Within each latent class, the model permitted intercept and slope to vary; these variances captured individual deviations from the mean trajectory in starting levels and rates of change. This flexibility reflected realistic variation in human behavior. The analysis fixed the covariance between intercept and slope at zero, so an individual's initial consumption level did not predict the rate of change. This restriction aided convergence and aligned with analysis aims. Removing the covariance term reduced parameters for estimation and supported model estimation with missing information from these repeated measures. Each class represented a distinct pattern of alcohol use across pregnancy. The Mplus syntax is in Supplemental Code Listing A.1.

Analysis of Baseline Characteristics

To illustrate the consumption patterns defining each class, mean (±SD) monthly winsorized alcohol intake (ozAA/day) was calculated for each trajectory group across gestation.

Baseline maternal demographic and clinical characteristics were compared across alcohol

trajectory groups. These characteristics included gestational age at enrollment, maternal age, gestational week at pregnancy recognition, pre-pregnancy BMI, total smoking during pregnancy, gravidity (total number of pregnancies), TWEAK score, maternal education (years), and past marijuana use. For continuous maternal variables, ANOVA tested for overall group differences, followed by pairwise comparisons of estimated marginal means with Benjamini-Hochberg (BH) adjustment for multiple comparisons. For the categorical variable of past marijuana use, logistic regression assessed group differences, with subsequent pairwise comparisons of estimated marginal proportions also adjusted using the BH procedure.

Infant characteristics at birth and neurodevelopmental outcomes were compared across the maternal alcohol consumption trajectory groups. Differences in these continuous infant variables across the five maternal alcohol trajectory groups were evaluated using ANOVA, followed by pairwise comparisons of estimated marginal means with BH adjustment for multiple comparisons.

Analysis of Maternal Biomarkers

A generalized linear mixed model (GLMM) modeled repeated biomarker measurements from early/mid and late gestation. This statistical approach used random effects to manage correlated observations from the same individuals and address nested measurements within subjects. The model structure combined these random effects with fixed effects (e.g., supplementation status, alcohol exposure pattern). The analysis used all available data, without requiring imputation. Models were estimated in R using `glmmTMB` (version 1.1.10)[62].

Preliminary analyses indicated that the additional choline supplementation, provided to a subset of participants receiving MVM, did not alter maternal biomarker outcomes beyond the effects of MVM supplementation alone. For this reason, maternal vitamin use groups were defined based on MVM intake (categorized as No MVM, MVM SE, or MVM PE), combining participants irrespective of choline co-intervention for the analyses presented.

Fixed effects in the GLMM comprised alcohol trajectory group, MVM use, gestational age

at measurement, pre-pregnancy BMI, interview location, marijuana use during pregnancy, maternal age, maternal education, SES, total smoking during pregnancy, and total number of pregnancies. The model included an interaction term between alcohol trajectory group and nutrient supplementation. Random-effects structures, incorporating random intercepts and slopes for gestational age grouped by mother, captured individual differences.

The analysis examined main effects of trajectory group and nutrient supplementation. Significant interactions between these factors obscured independent interpretation of their main effects. Estimated marginal means were calculated to clarify these joint effects. These means illustrated the joint influences of nutrient supplementation and exposure group on biomarker outcomes.

Analysis of Infant Outcomes

To investigate the potential modifying effect of maternal MVM supplementation on the association between PAE trajectories and infant outcomes, a series of generalized linear model (GLM) statistics were fitted using the `glmmTMB` package in R (version 1.1.10). Separate models were constructed for each infant outcome: birthweight, length, head circumference, and MDI and PDI scores at 6 and 12 months.

For each outcome, models included main effects for alcohol trajectory group and MVM supplementation status, as well as their interaction term. Covariates included maternal age, SES, total smoking during pregnancy, and gestational age at enrollment. For models demonstrating significant main effects or interactions, post-hoc analyses of estimated marginal means were conducted with BH adjustment for multiple comparisons to examine differences between alcohol trajectory groups overall and within each MVM supplementation status, as well as differences between MVM supplementation statuses within each alcohol trajectory group.

To account for multiple comparisons, we applied the Benjamini-Hochberg procedure to control the False Discovery Rate (FDR)[63]. This method, which adjusts p-values based on

their rank, was selected over the Bonferroni correction to mitigate the increased risk of Type II errors associated with the Bonferroni approach and to avoid overcorrecting non-significant results[64]. Both uncorrected p-values and FDR-adjusted q-values are presented. Results were considered statistically significant at an FDR-adjusted q-value of < 0.05.

3.3 Results

3.3.1 Prenatal Alcohol Trajectory Groups

Models with two to six classes were compared to determine the best fit. Selection criteria included the akaike information criterion (AIC), bayesian information criterion (BIC), parsimony, entropy, and alignment with theoretical expectations. Participants were assigned to trajectory subgroups based on their highest posterior probability of membership.

The four- and five-class solutions performed better than models with fewer or more classes (Table 3.1). The five-class solution had the lowest adjusted BIC (886.341). Its improvement over the four-class solution (adjusted BIC = 948.980) was modest considering parsimony. The change in BIC was -88.347 for the four-class model and -62.639 for the five-class model, suggesting limited incremental benefit from an additional class. A six-class model yielded the highest entropy (0.810), indicating a slight gain in classification accuracy, but its overall fit improved minimally over the four-class model (entropy = 0.786).

A four-class solution was selected. This solution balanced AIC, BIC, and changes in BIC, and aligned with trajectories from previous studies of this population[37,38]. Individual prenatal alcohol consumption trajectories (Figure 3.1) and latent class averages (Figure 3.2) were classified as Abstained (Abstain), Low Sustained (Low), Moderate-to-Discontinued/Low Sustained (ModDisc), High-to-Moderate Sustained (HighMod), and Very High Sustained (VHigh).

The identified trajectories correspond well with those reported by Bandoli et al. (2019) in a similar cohort. Both analyses identified a very high sustained trajectory that remained

Table 3.1: Fit statistics for two to six latent class growth mixture models of pregnancy alcohol trajectories

k	Log-likelihood	AIC	BIC	Entropy	ΔBIC
2	-504.927	1039.854	1097.200	0.809	-667.131
3	-460.433	960.867	1037.327	0.754	-59.873
4	-401.702	853.404	948.980	0.786	-88.347
5	-355.825	771.649	886.341	0.792	-62.639
6	-346.264	762.527	896.334	0.810	9.993

Columns display the number of classes (k), AIC, BIC, entropy, and the change in BIC (ΔBIC) relative to the model with one fewer class.

elevated above all other groups throughout gestation. Each study found two trajectories characterized by moderate-to-high intake that gradually reduced during pregnancy, and one trajectory with consistent, sustained alcohol consumption. The overall patterns were comparable while the average daily alcohol intake (ozAA/day) for corresponding trajectories differed slightly between the two studies.

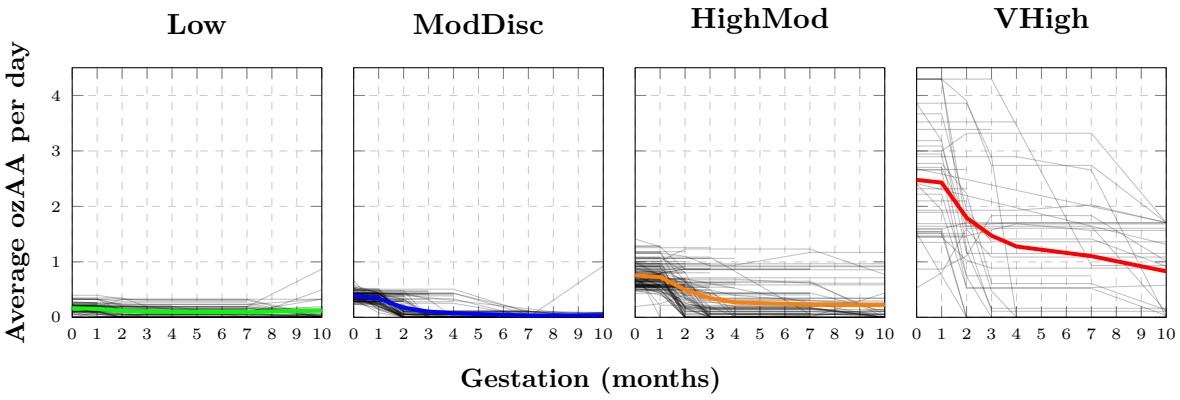


Figure 3.1: Prenatal alcohol consumption patterns for the four identified latent classes. Each panel displays individual participant trajectories (black lines) and the mean trajectory (color line) for a specific class in ounces of absolute alcohol (ozAA) per day across gestation.

3.3.2 Maternal and Infant Characteristics

Table 3.2 details maternal characteristics across prenatal alcohol exposure trajectory groups. Participants in the HighMod trajectory enrolled later in gestation than the Abstain group.

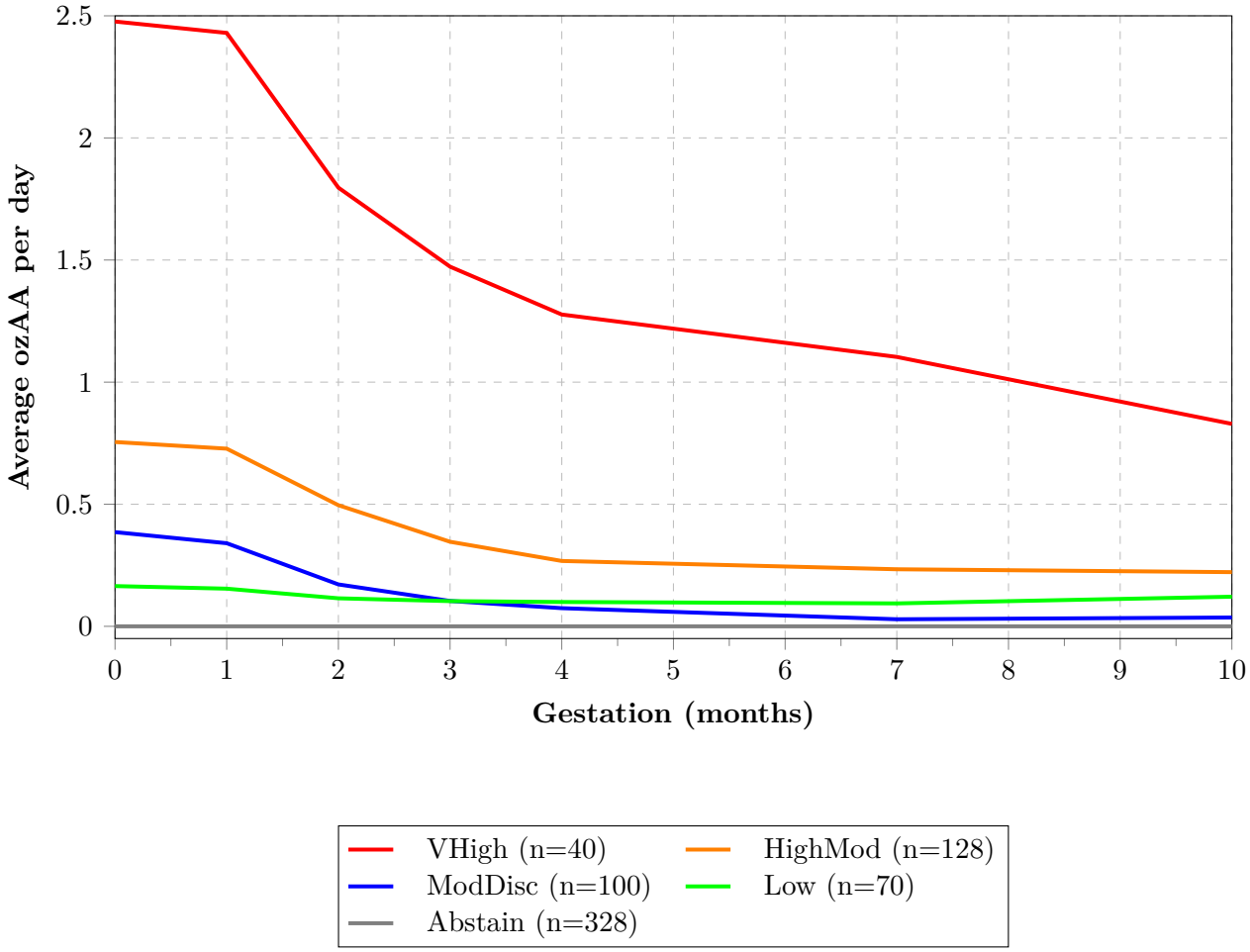


Figure 3.2: Mean prenatal alcohol consumption trajectories (ounces of absolute alcohol [ozAA]/day) for the four identified latent classes and the Abstained group.

The gestational week at pregnancy recognition progressively increased with higher alcohol consumption trajectories; the VHigh group recognized pregnancy latest, differing from the Abstain, Low, and ModDisc groups. The VHigh group reported higher gravidity than the Abstain and ModDisc groups. Maternal education years decreased with increasing alcohol exposure; the Abstain group reported more education years than all other groups, and the HighMod and VHigh groups reported the fewest. The HighMod group reported a higher proportion of maternal marijuana use than the Abstain group. Daily cigarette consumption and TWEAK scores increased progressively across trajectories from Abstain to VHigh. For TWEAK scores, all groups differed from one another with the Abstain group having the

Table 3.2: Maternal and Infant Characteristics by Prenatal Alcohol Exposure Trajectory Group

Characteristic	Abstain (N=328)	Low (N=70)	ModDisc (N=99)	HighMod (N=128)	VHigh (N=40)	p-value
Maternal age (years)	26.2 (4.60)	26.0 (5.77)	25.8 (5.67)	25.8 (5.88)	27.7 (6.13)	0.360
Gestational week at enrollment	17.2 (5.85)^{a}	18.3 (5.62)^{ab}	18.9 (6.53)^{ab}	20.3 (7.57)^{b}	19.2 (7.21)^{ab}	<0.001
Gestational week at pregnancy recognition	5.06 (2.67)^{a}	5.75 (3.68)^{ab}	6.73 (4.74)^{b}	6.74 (3.87)^{b}	8.45 (5.06)^{c}	<0.001
Gestational age at birth (weeks)	39.3 (2.98)	39.2 (2.75)	38.8 (3.11)	38.7 (3.41)	39.0 (1.95)	0.402
Gravidity (number of other pregnancies)	2.05 (1.38)^{a}	2.31 (1.60)^{ab}	1.94 (1.40)^{a}	2.24 (1.79)^{a,b}	2.80 (1.70)^{b}	0.019
Maternal prepregnancy BMI	22.0 (3.72)	22.6 (4.22)	22.0 (3.74)	22.5 (3.62)	22.3 (3.50)	0.511
Maternal education (years)	14.6 (2.22)^{a}	13.1 (2.60)^{bc}	13.3 (2.43)^{b}	12.2 (2.45)^{d}	12.2 (2.56)^{cd}	<0.001
Maternal marijuana usage, n (%)	1 (0.3)^{a}	1 (1.4)^{ab}	3 (3.0)^{ab}	9 (7.0)^{b}	1 (2.5)^{ab}	0.001
Cigarettes smoked per day (n)	0.18 (1.36)^{a}	0.83 (2.21)^{ab}	1.17 (4.03)^{b}	2.37 (4.82)^{c}	2.58 (3.90)^{c}	<0.001
TWEAK score	0.05 (0.38)^{a}	1.21 (1.28)^{b}	1.73 (1.28)^{c}	2.30 (1.49)^{d}	2.87 (2.02)^{e}	<0.001
Infant birthweight (g)	3357 (556)^{a}	3277 (542)^{ab}	3182 (616)^{b}	3128 (608)^{b}	3015 (521)^{b}	<0.001
Infant length (cm)	51.7 (2.99)	51.6 (2.89)	50.9 (3.71)	50.8 (3.40)	50.5 (3.19)	0.018
Infant head circumference (cm)	34.4 (2.05)^{a}	34.2 (1.56)^{ab}	34.0 (2.03)^{ab}	33.8 (1.95)^{b}	33.6 (1.98)^{ab}	0.009
6 months Bayley MDI score	90.5 (7.02)^{a}	89.7 (6.85)^{a}	90.9 (10.1)^{a}	88.4 (9.78)^{a}	80 (12.9)^{b}	<0.001
12 months Bayley MDI score	91.5 (10.6)^{a}	86.8 (12.2)^{b}	89.6 (12.8)^{ab}	88.3 (12.8)^{ab}	80.8 (15.1)^{b}	<0.001
6 months Bayley PDI score	89.5 (10.2)^{a}	86.6 (11.8)^{a}	90.3 (13.7)^{a}	88.7 (12.1)^{a}	79.7 (14.2)^{b}	<0.001
12 months Bayley PDI score	99.8 (11.8)^{a}	94.5 (14.5)^{b}	96.7 (11.9)^{ab}	96.5 (15.3)^{ab}	89.3 (16.4)^{b}	<0.001

Values are presented as mean (standard deviation) for continuous variables or n (%) for categorical variables. Overall p-values assess differences across groups using ANOVA for continuous variables and logistic regression for categorical variables. Within a row, values not sharing a common superscript letter are statistically different ($p < 0.05$) based on pairwise comparisons with BH adjustment.

lowest and the VHigh group having the highest TWEAK score. Maternal age, BMI prior to pregnancy, and gestational age at birth did not differ significantly among the trajectory groups.

3.3.3 Maternal Markers of Oxidative Stress and Antioxidant Defense

Indicators of Oxidative Damage and Global Antioxidant Capacity

TBARS (Thiobarbituric Acid Reactive Substances) TBARS levels showed a quadratic relationship with alcohol trajectory categories ($\beta = 0.126$; SE = 0.034; $p < 0.001$). Participants in the MVM SE group ($\beta = –0.076$; SE = 0.023; $p < 0.001$) and the MVM PE group ($\beta = –0.106$; SE = 0.026; $p < 0.0001$) had lower TBARS levels than participants in the No MVM group.

Within the Abstain group, participants in the No MVM group (1.498 ± 0.051 µM MDA)

had higher TBARS levels compared with participants in the MVM SE group (1.334 ± 0.048 µM MDA; $p < 0.001$; $q = 0.001$) or the MVM PE group (1.285 ± 0.044 µM MDA; $p < 0.0001$; $q < 0.0001$) (Figure 3.3). A comparable pattern occurred in the VHigh group: participants in the No MVM group (1.615 ± 0.082 µM MDA) had higher TBARS levels than those in the MVM SE group (1.348 ± 0.081 µM MDA; $p = 0.025$; $q = 0.163$); this difference was not significant after correction.

Among participants in the No MVM group, the VHigh subgroup exhibited higher TBARS (1.615 ± 0.082 µM MDA) than the Low subgroup (1.409 ± 0.062 µM MDA; $p = 0.014$; $q = 0.106$), the ModDisc subgroup (1.352 ± 0.059 µM MDA; $p = 0.001$; $q = 0.021$), and the HighMod subgroup (1.420 ± 0.054 µM MDA; $p = 0.012$; $q = 0.106$) (Figure 3.4). Only the comparison with the ModDisc group remained significant after post-hoc adjustment.

MVM supplementation attenuated these differences. For participants in the MVM SE group, those in the VHigh group had TBARS levels comparable to other alcohol trajectory groups. Among participants in the MVM PE group, those in the VHigh group showed higher TBARS levels only when compared to individuals in the ModDisc group (1.457 ± 0.120 vs. 1.225 ± 0.058 µM MDA; $p = 0.044$, $q = 0.250$); this difference was not significant after BH correction.

TRAP (Total Radical-Trapping Antioxidant Parameter) Longitudinal analysis of TRAP varied by the linear ($\beta = 0.067$; SE = 0.034; $p = 0.047$) and cubic ($\beta = –0.062$; SE = 0.031; $p = 0.044$) polynomial contrasts of Alcohol Trajectory. No main effects emerged for MVM SE or MVM PE. TRAP values were log-transformed for modeling, then back-transformed to obtain the adjusted means.

Among No MVM participants, the Low group displayed lower TRAP levels relative to other trajectory groups; these comparisons were not significant after adjustment (Figure 3.5). Among MVM PE participants, the VHigh group (160.453 ± 1.082 µM) had elevated TRAP levels compared to the Abstain group (136.721 ± 1.032 µM; $p = 0.0385$; $q = 0.288$)

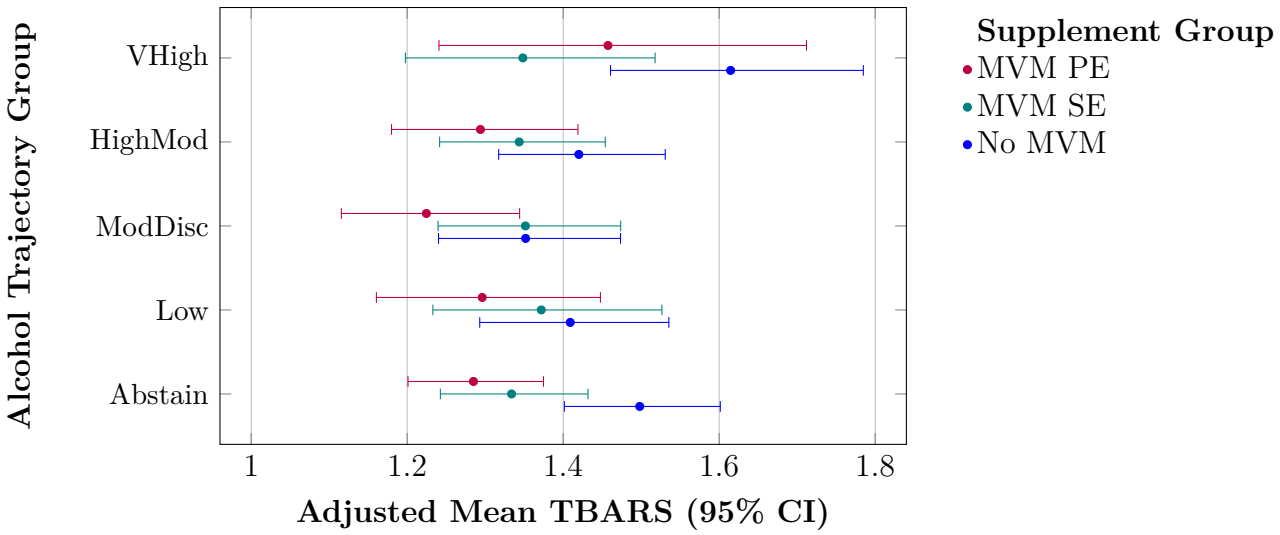


Figure 3.3: Forest plot of adjusted mean plasma TBARS (µM MDA) levels with 95% confidence intervals. Values are displayed across maternal alcohol consumption trajectories (Abstain; Low; ModDisc; HighMod; VHigh) within each MVM supplementation group (No MVM; MVM SE; MVM PE). Estimates are from a GLMM.

and the ModDisc group (134.879 ± 1.044 µM; p = 0.0370; q = 0.288).

Enzymatic Antioxidant System Components

SOD (Superoxide Dismutase) SOD activity did not differ among the Alcohol Trajectory groups. Participants in the MVM SE group (β = 31.722; SE = 13.723; p = 0.021) and the MVM PE group (β = 48.200; SE = 15.776; p = 0.002) exhibited higher SOD activity compared to the No MVM group.

Within the VHigh alcohol trajectory group, participants in the No MVM group (832.897 ± 31.488 U/g Hb) showed lower SOD activity compared to those in the MVM SE group (941.190 ± 35.577 U/g Hb; p = 0.031; q = 0.232) and the MVM PE group (1024.551 ± 49.498 U/g Hb; p = 0.001; q = 0.044) (Figure 3.6). The difference in SOD activity between the No MVM and MVM SE subgroups within the VHigh trajectory lost significance after adjustment. In alcohol trajectory groups other than VHigh, SOD activity did not differ among the three MVM supplementation statuses.

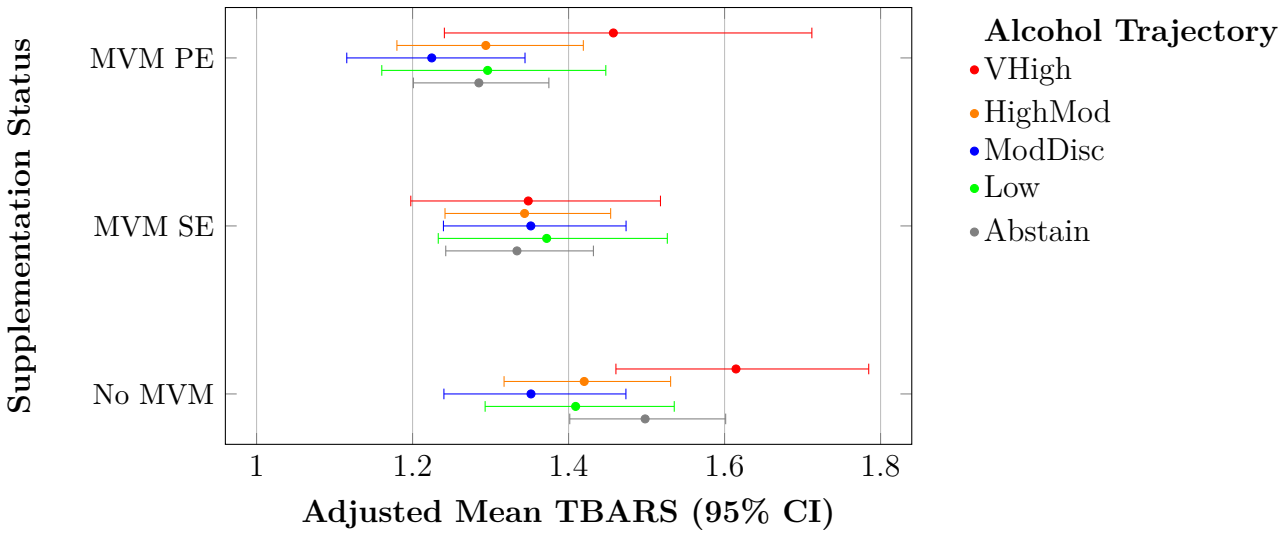


Figure 3.4: Forest plot of adjusted mean plasma TBARS (µM MDA) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups (No MVM; MVM SE; MVM PE) within each maternal alcohol consumption trajectory (Abstain; Low; ModDisc; HighMod; VHigh). Estimates are from a GLMM.

Among participants in the MVM SE group, those in the VHigh alcohol trajectory exhibited significantly higher SOD activity than participants in the HighMod trajectory (864.709 ± 23.006 U/g Hb; p = 0.041; q=0.265); this difference was no longer significant after adjustment (Figure 3.7). A similar pattern was observed in the MVM PE group. Within the MVM PE group, participants in the VHigh trajectory demonstrated higher SOD activity compared to those in the Abstain (880.398 ± 19.439 U/g Hb; p = 0.003; q = 0.044), Low (874.094 ± 32.148 U/g Hb; p = 0.006; q = 0.060), ModDisc (883.225 ± 26.489 U/g Hb; p = 0.007; q = 0.060), and HighMod (864.728 ± 26.029 U/g Hb; p = 0.003; q = 0.044) alcohol trajectory groups.

GRed (Glutathione Reductase) GRed levels varied by the quadratic term of Alcohol Trajectory (β = -0.093; SE = 0.041; p = 0.025). GRed levels were higher in the MVM SE group (β = 0.070; SE = 0.027; p = 0.009) and the MVM PE group (β = 0.132; SE = 0.031; p < 0.0001) compared to the No MVM group. No significant interactions between Alcohol Trajectory and MVM status were observed.

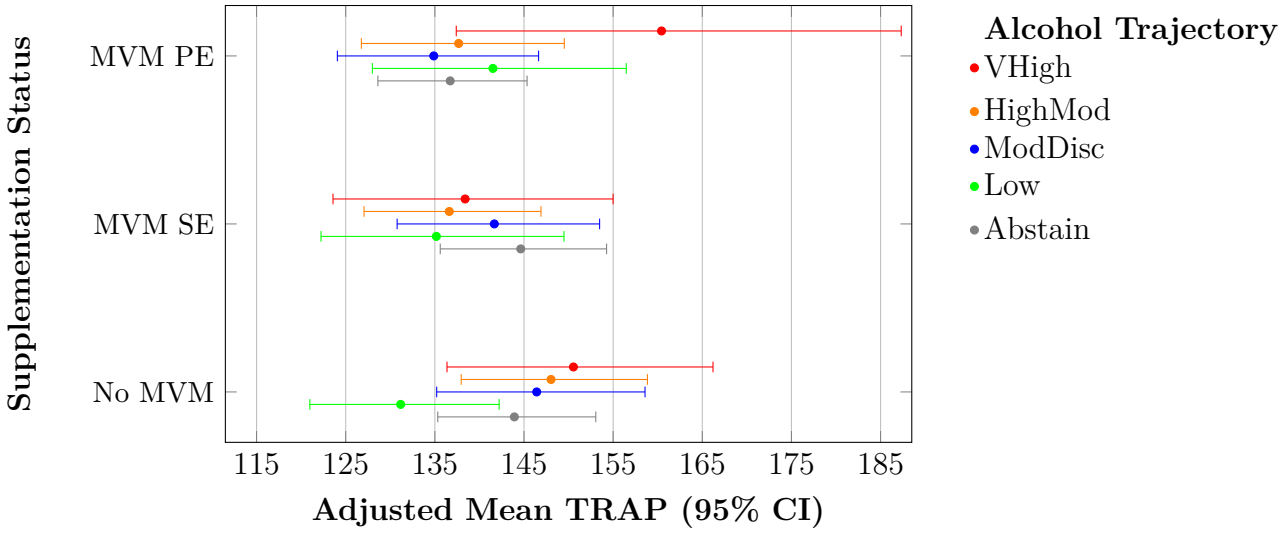


Figure 3.5: Forest plot of adjusted mean plasma TRAP (µM) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups within each maternal alcohol consumption trajectory. Estimates are from a GLMM.

Comparisons among the three supplementation groups showed that GRed levels were higher in both the MVM SE group (0.852 ± 0.029 µmol NADPH/min/g Hb; p = 0.023; q = 0.152) and the MVM PE group (0.852 ± 0.029 µmol NADPH/min/g Hb; p < 0.0001; q = 0.001) than in the No MVM group (0.794 ± 0.026 µmol NADPH/min/g Hb) (Figure 3.8). This pattern remained consistent across Alcohol Trajectory groups. Since no significant interaction emerged, differences were not separately tested for each trajectory group.

GPx (Glutathione Peroxidase) GPx activity did not differ across the Alcohol Trajectories. MVM SE and MVM PE showed no association with GPx activity. Data not shown.

3.3.4 Maternal Markers of Acute Phase Response and Nutrient Homeostasis

Inflammation and Acute Phase Protein Indicators

hsCRP (High-Sensitivity C-Reactive Protein) Log-transformed hsCRP levels varied across alcohol trajectory groups, exhibiting a quadratic pattern (β = 0.218; SE = 0.075; p

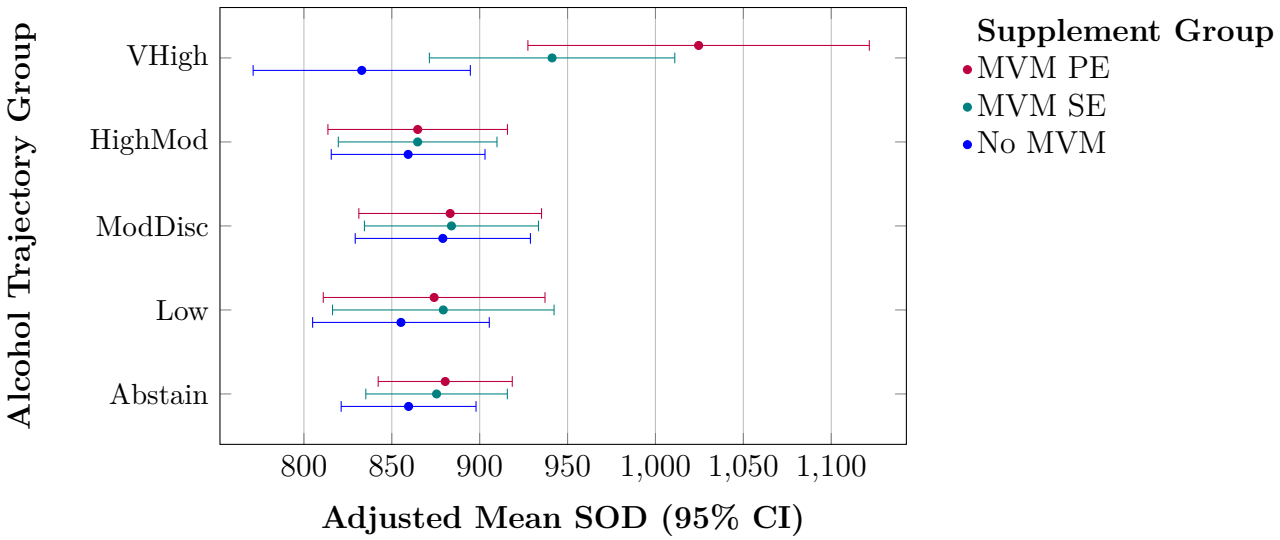


Figure 3.6: Forest plot of adjusted mean erythrocyte SOD (U/g Hb) levels with 95% confidence intervals. Values are displayed across maternal alcohol consumption trajectories within each MVM supplementation group. Estimates are from a GLMM.

= 0.004). MVM PE had a borderline association with lower hsCRP levels than participants in the No MVM group (β = -0.117; SE = 0.069; p = 0.090). All hsCRP values were log-transformed for modeling, then back-transformed to obtain adjusted means.

Within the No MVM group, the Abstain group had higher hsCRP levels (3.958 ± 1.109 mg/L) than the Low group (3.144 ± 1.120 mg/L; p = 0.041; q = 0.265) (Figure 3.9). The VHigh group exhibited elevated hsCRP (4.651 ± 1.192 mg/L) relative to the Low group (p = 0.020; q = 0.178) and the HighMod group (3.284 ± 1.107 mg/L; p = 0.027; q = 0.200). These differences were not significant after multiple comparison correction.

Participants in the VHigh trajectory within the MVM PE group had lower hsCRP levels (2.004 ± 1.194 mg/L) than participants in the Abstain group (3.748 ± 1.104 mg/L; p = 0.011; q = 0.164), the Low group (4.140 ± 1.183 mg/L; p = 0.008; q = 0.164), and the ModDisc group (3.674 ± 1.141 mg/L; p = 0.017; q = 0.178) (Figure 3.10). These contrasts were not significant after adjustment.

Within the VHigh group, MVM PE was associated with lower hsCRP than the No MVM group (2.004 vs. 4.651 mg/L; p = 0.008; q = 0.164) (Figure 3.10). MVM SE was associated

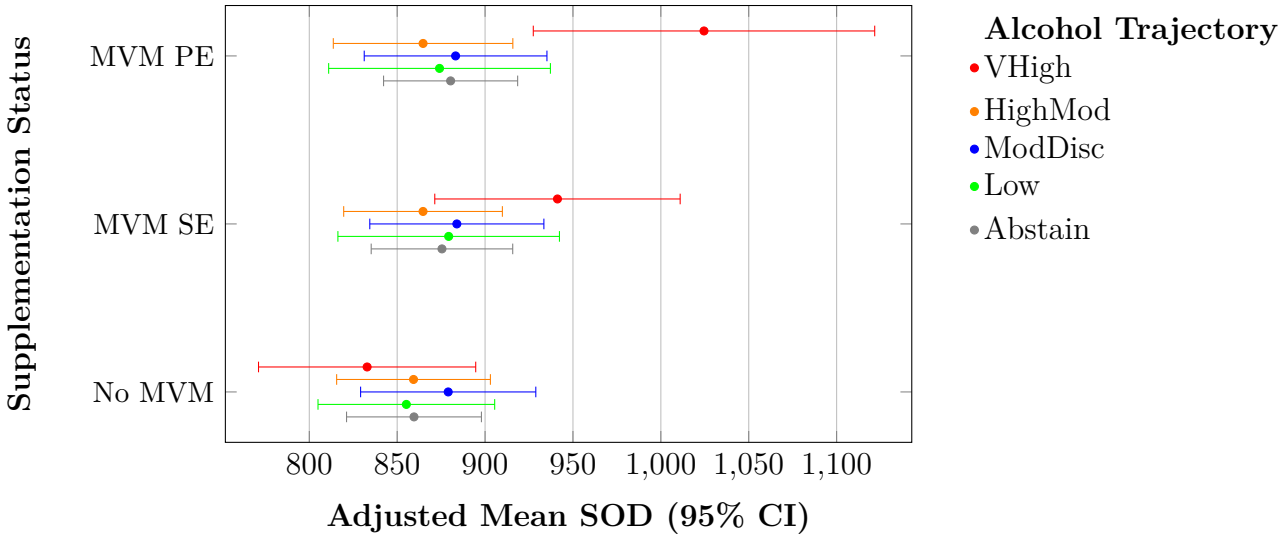


Figure 3.7: Forest plot of adjusted mean erythrocyte SOD (U/g Hb) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups within each maternal alcohol consumption trajectory. Estimates are from a GLMM.

with a lesser reduction (2.910 ± 1.192 vs. 4.651 mg/L; p = 0.057; q = 0.265), which was not statistically significant.

CP (Ceruloplasmin) CP levels did not differ across Alcohol Trajectories or MVM supplementation groups. Data not shown.

Plasma Trace Mineral Levels

Zinc Plasma zinc levels varied according to linear (β = –0.130; SE = 0.033; p < 0.0001) and quadratic (β = –0.161; SE = 0.031; p < 0.0001) polynomial contrasts for the alcohol trajectory classification. Participants in the MVM SE group had higher zinc levels than the No MVM group (β = 0.102; SE = 0.019; p < 0.0001). Elevated zinc levels were observed in the MVM PE group (β = 0.063; SE = 0.024; p = 0.009) compared to the No MVM group.

Within the No MVM group, the VHigh trajectory (0.468 ± 0.023 µg/mL) exhibited lower zinc levels compared with the Abstain (0.563 ± 0.015 µg/mL; p < 0.0001; q = 0.001), Low (0.608 ± 0.021 µg/mL; p < 0.0001; q < 0.0001), ModDisc (0.597 ± 0.020 µg/mL; p < 0.0001;

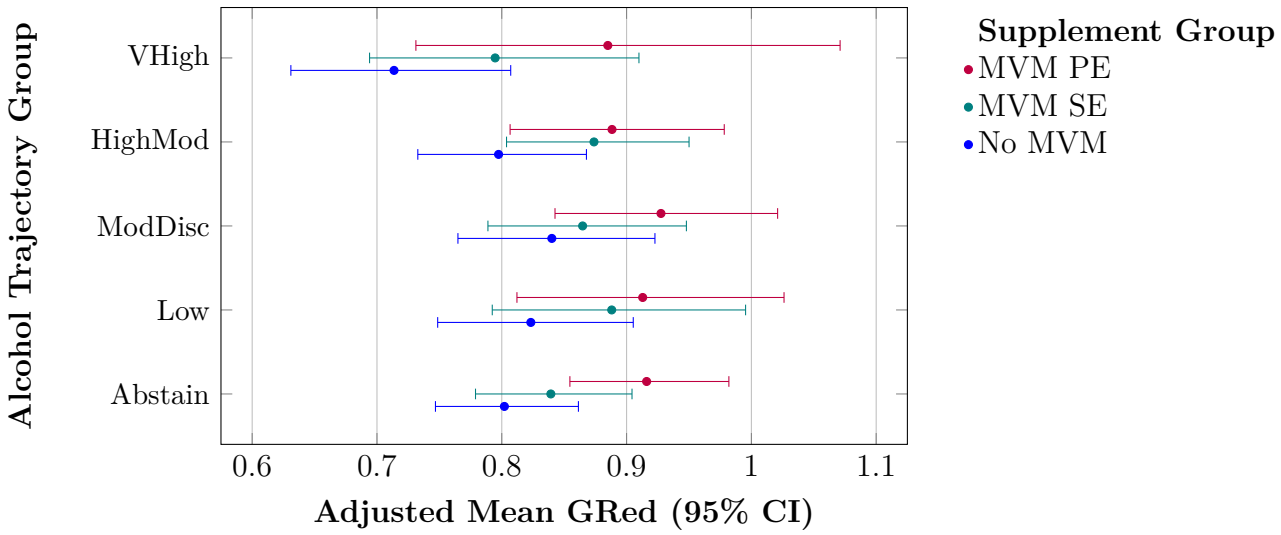


Figure 3.8: Forest plot of adjusted mean erythrocyte GRed (µmol NADPH/min/g Hb) levels with 95% confidence intervals. Values are displayed across maternal alcohol consumption trajectories within each MVM supplementation group. Estimates are from a GLMM.

$q < 0.0001$), and HighMod (0.584 ± 0.018 µg/mL; $p < 0.0001$; $q < 0.001$) trajectories (Figure 3.11). This pattern was not observed among nutrient supplemented participants.

Within the Abstain trajectory, participants in the No MVM group (0.563 ± 0.015 µg/mL) had lower zinc levels than those in the MVM SE group (0.632 ± 0.017 µg/mL; $p < 0.0001$; $q < 0.0001$) or the MVM PE group (0.617 ± 0.016 µg/mL; $p < 0.0001$; $q < 0.001$) (Figure 3.12).

In the HighMod trajectory, participants in the No MVM group (0.584 ± 0.018 µg/mL) had lower zinc levels than participants in the MVM SE group (0.636 ± 0.019 µg/mL; $p = 0.030$; $q = 0.149$) (Figure 3.12). Within the VHigh trajectory, participants in the No MVM group (0.468 ± 0.023 µg/mL) had lower zinc levels than those in the MVM SE group (0.600 ± 0.029 µg/mL; $p < 0.001$; $q = 0.002$); this latter comparison retained significance after multiple-comparison adjustment.

Copper Plasma copper levels exhibited a linear ($\beta = -0.096$; SE = 0.033; $p = 0.004$) and quadratic ($\beta = -0.075$; SE = 0.033; $p = 0.022$) relationship with alcohol trajectory patterns.

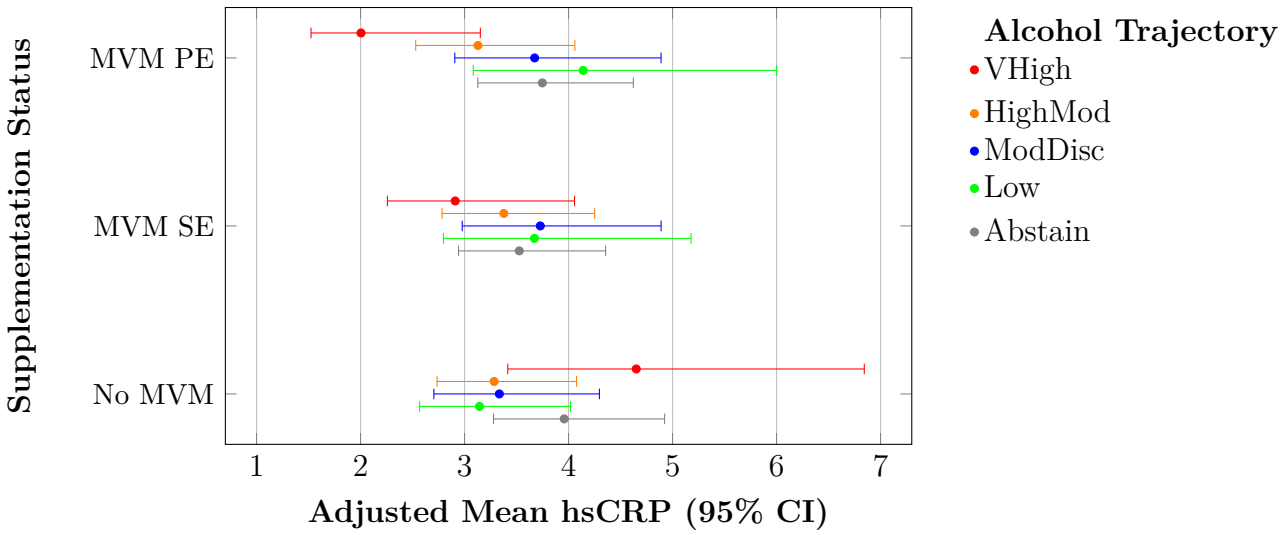


Figure 3.9: Forest plot of adjusted mean plasma hsCRP (mg/L) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups within each maternal alcohol consumption trajectory. Estimates are from a GLMM.

Participants in the MVM SE group demonstrated elevated copper levels compared to No MVM participants ($\beta = 0.079$; SE = 0.021; $p < 0.001$). MVM PE participants displayed a non-significant trend toward higher copper ($\beta = 0.049$; SE = 0.025; p = 0.051).

Within the Abstain group, MVM SE participants (1.776 ± 0.057 µg/mL) had higher copper levels than their No MVM counterparts (1.627 ± 0.050 µg/mL; p = 0.002; q = 0.036) (Figure 3.13). MVM PE participants (1.743 ± 0.053 µg/mL) exhibited a comparable elevation over No MVM participants (p = 0.012; q = 0.078); this difference was no longer significant after multiple-test adjustment.

Comparable findings were observed in the HighMod group (Figure 3.13). MVM SE participants (1.766 ± 0.063 µg/mL; p = 0.001; q = 0.036) and MVM PE participants (1.784 ± 0.073 µg/mL; p = 0.006; q = 0.075) both had higher copper levels than No MVM individuals (1.548 ± 0.054 µg/mL). VHigh participants in the MVM SE group showed a marginal elevation over their No MVM counterparts (1.708 ± 0.095 vs 1.458 ± 0.073 µg/mL; p = 0.051; q = 0.164).

Among No MVM participants, the VHigh group exhibited lower copper than Abstain

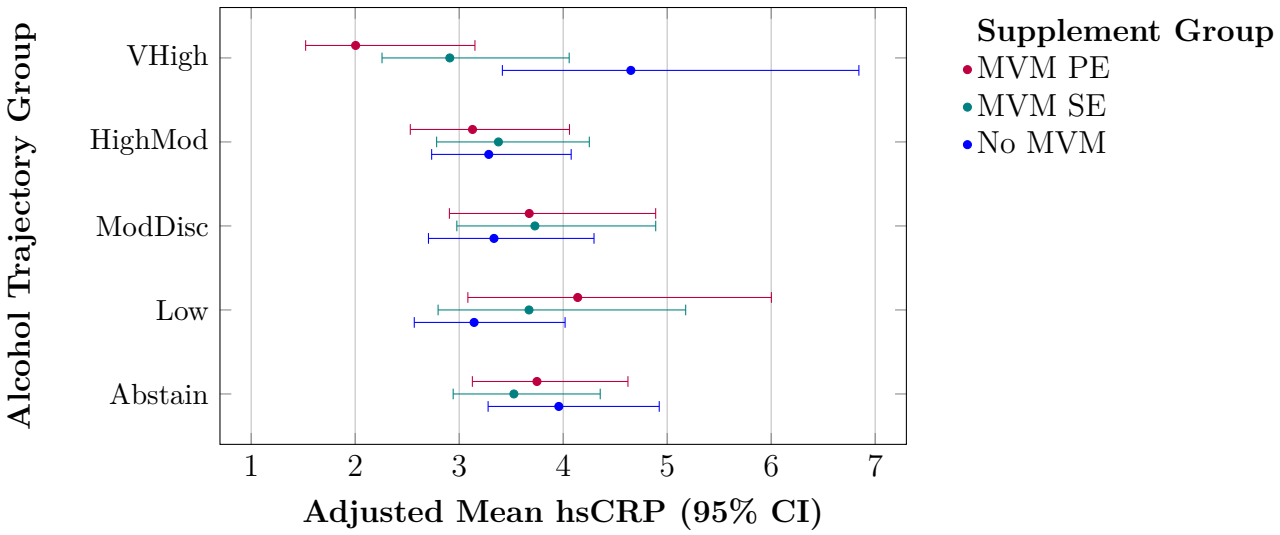


Figure 3.10: Forest plot of adjusted mean plasma hsCRP (mg/L) levels with 95% confidence intervals. Values are displayed across maternal alcohol consumption trajectories within each MVM supplementation group. Estimates are from a GLMM.

participants (1.458 ± 0.073 vs 1.627 ± 0.050 µg/mL; p = 0.022; q = 0.099), the Low group (1.682 ± 0.067 µg/mL; p = 0.009; q = 0.078), and the ModDisc group (1.692 ± 0.067 µg/mL; p = 0.007; q = 0.075) (Figure 3.14). A comparable pattern was observed in the MVM PE group, where the VHigh group (1.432 ± 0.117 µg/mL) had lower copper levels than the Abstain group (1.743 ± 0.053 µg/mL; p = 0.015; q = 0.082), the Low group (1.762 ± 0.088 µg/mL; p = 0.021; q = 0.099), the ModDisc group (1.704 ± 0.071 µg/mL; p = 0.042; q = 0.156), and the HighMod group (1.784 ± 0.073 µg/mL; p = 0.012; q = 0.078), with marginal significance after adjustment.

Iron Plasma iron levels differed significantly across alcohol trajectory groups, following a quadratic association (β = –0.126; SE = 0.054; p = 0.020). Participants in the MVM SE group showed a trend of higher iron levels than individuals in the No MVM group; this trend was not statistically significant (β = 0.061; SE = 0.037; p = 0.097).

In the No MVM group, participants in the Abstain group had lower iron levels (0.768 ± 0.041 µg/mL) than those in the ModDisc group (0.871 ± 0.059 µg/mL; p = 0.041; q =

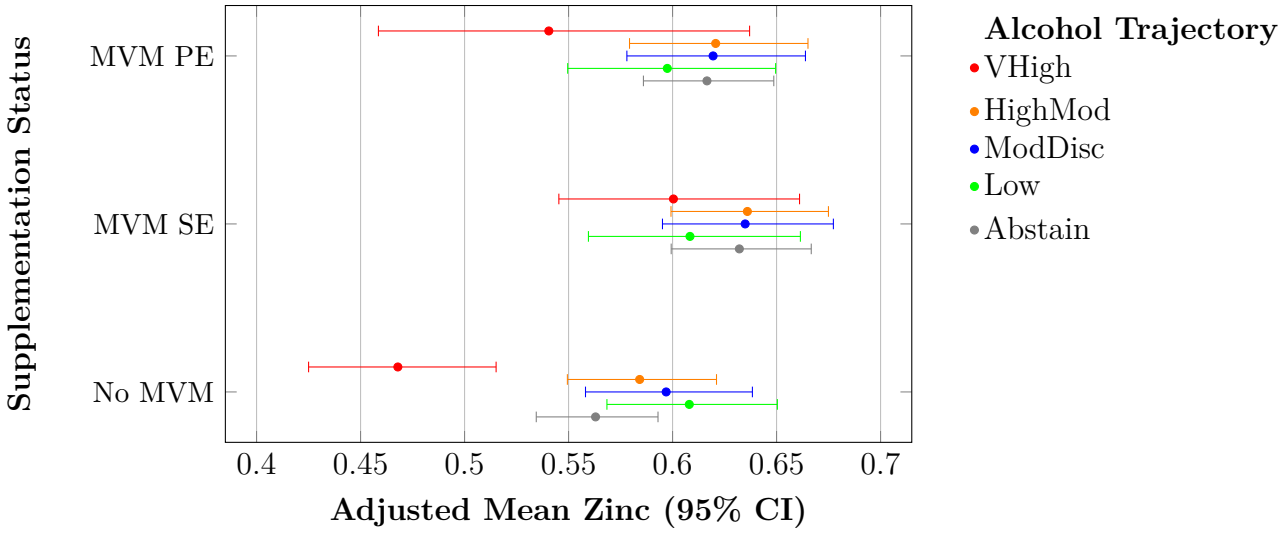


Figure 3.11: Forest plot of adjusted mean plasma zinc (µg/mL) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups within each maternal alcohol consumption trajectory. Estimates are from a GLMM.

0.578), however, this difference was not present after correction for multiple comparisons (Figure 3.14). Plasma iron concentrations did not differ significantly among other trajectory groups.

Iron Status Indicators

Ferritin Plasma ferritin levels displayed a linear association with alcohol trajectory (β = 0.096; SE = 0.034; p = 0.006). Neither the MVM SE group nor the MVM PE group demonstrated significant effects. Plasma ferritin values were log-transformed for modeling and back-transformed for the adjusted means.

In the No MVM group, the Abstain group (18.323 ± 1.101 ng/mL) showed lower ferritin compared to the ModDisc (25.711 ± 1.145 ng/mL; p = 0.005; q = 0.074), HighMod (25.573 ± 1.127 ng/mL; p = 0.002; q = 0.045), and VHigh (25.163 ± 1.180 ng/mL; p = 0.035; q = 0.169) groups (Figure 3.16). The difference with the HighMod group remained significant after multiple-comparison correction. The Low group (19.041 ± 1.137 ng/mL) exhibited lower ferritin levels than the ModDisc group (p = 0.048; q = 0.195) and the HighMod group

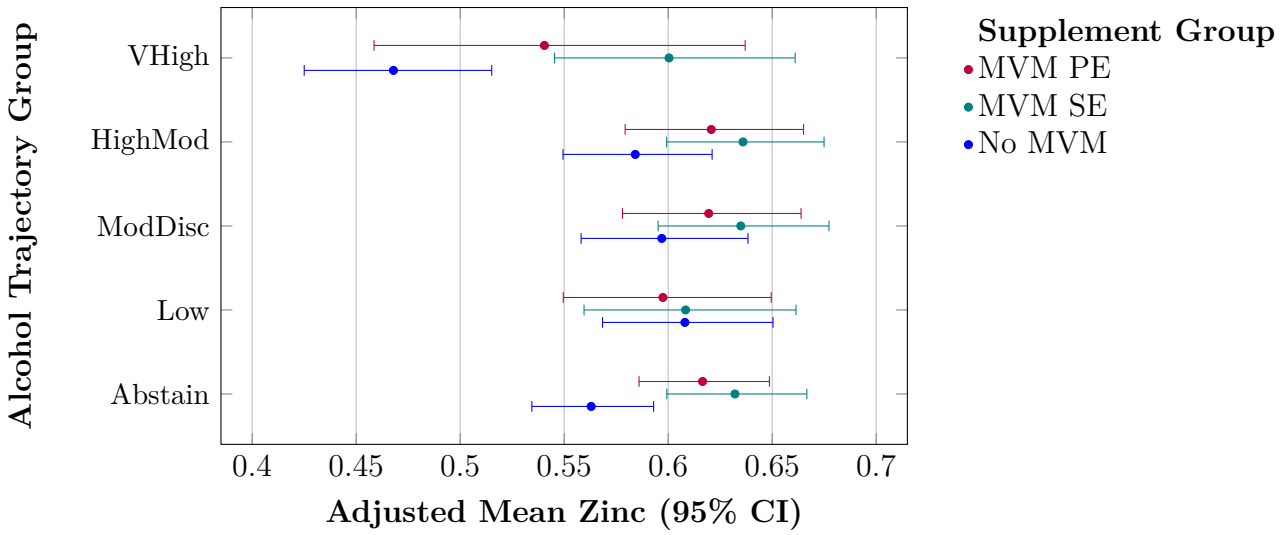


Figure 3.12: Forest plot of adjusted mean plasma zinc (µg/mL) levels with 95% confidence intervals. Values are displayed across maternal alcohol consumption trajectories within each MVM supplementation group. Estimates are from a GLMM.

(p = 0.036; q = 0.169) before adjustment for multiple measures.

A comparable pattern appeared in the MVM SE group. Here, the Abstain group (19.021 ± 1.108 ng/mL) was statistically lower than the HighMod group (27.726 ± 1.134 ng/mL; p = 0.001, q = 0.045) (Figure 3.16). Differences were found between the Abstain group and both the ModDisc group (24.593 ± 1.144 ng/mL; p = 0.036, q = 0.169) and the VHigh group (29.197 ± 1.215 ng/mL; p = 0.022, q = 0.169), though neither contrast persisted after correction. The Low group (17.954 ± 1.171 ng/mL) had lower ferritin levels than the HighMod group (p = 0.012, q = 0.137) and the VHigh group (p = 0.031, q = 0.169) before correction. These patterns were not observed in the MVM PE group.

TfR (Transferrin Receptor) Plasma TfR values did not vary across the Alcohol Trajectory categories. Participants in the MVM SE or MVM PE groups showed TfR values similar to those in the No MVM group.

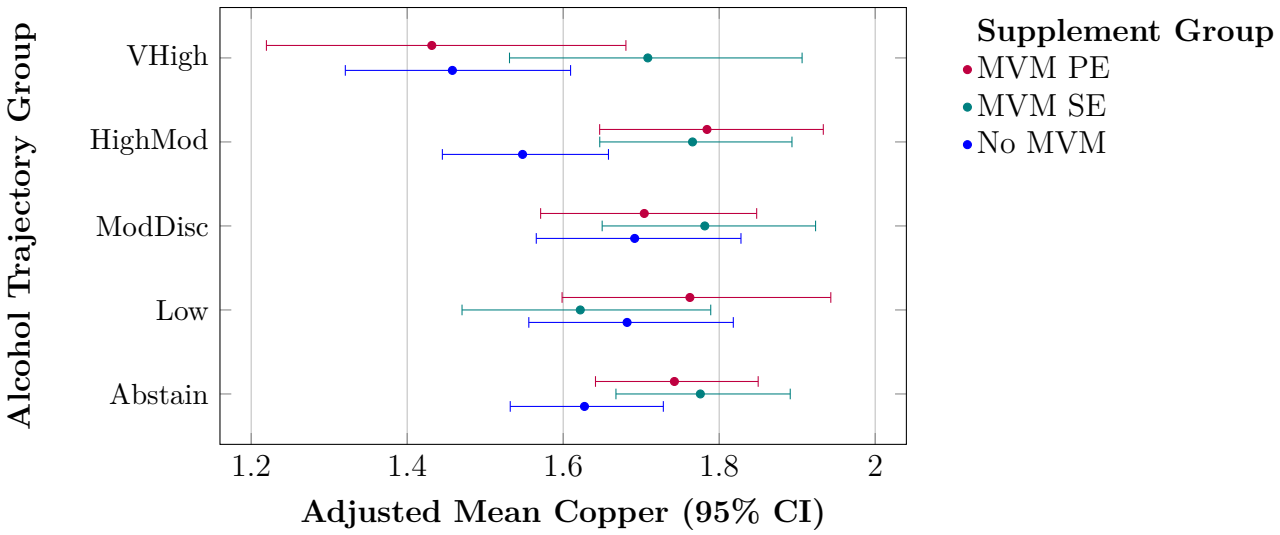


Figure 3.13: Forest plot of adjusted mean plasma copper (µg/mL) levels with 95% confidence intervals. Values are displayed across maternal alcohol consumption trajectories within each MVM supplementation group. Estimates are from a GLMM.

3.3.5 Impact of Alcohol Trajectories and MVM on Infant Outcomes

Physical Growth Parameters

GLM examined the impact of alcohol trajectories and maternal MVM use on infant physical growth and neurodevelopmental scores. For infant birth weight and length, models showed no significant main effects for alcohol trajectory or MVM status.

Infant Head Circumference A negative linear association occurred between alcohol trajectory group and infant head circumference (β = -0.646; SE = 0.309; p = 0.037); head circumference was smaller with higher alcohol exposure trajectories. An interaction between alcohol trajectory and MVM status for infant head circumference was not detected.

Pairwise comparisons of estimated marginal means for alcohol trajectory groups identified group differences. Infants in the VHigh group had a smaller head circumference (33.700 ± 0.277 cm) than infants in the Abstain group (34.500 ± 0.093 cm; p = 0.004, q = 0.035) (Figure 3.17). The VHigh group (33.700 ± 0.277 cm) also had a smaller head circumference than

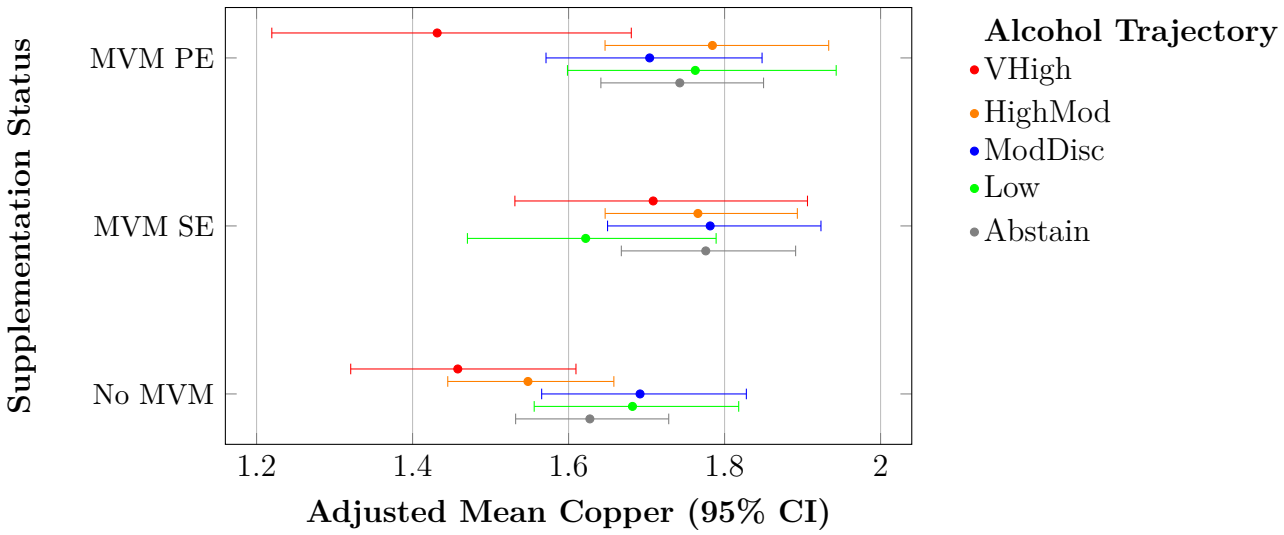


Figure 3.14: Forest plot of adjusted mean plasma copper (µg/mL) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups within each maternal alcohol consumption trajectory. Estimates are from a GLMM.

the Low group (34.300 ± 0.194 cm; p = 0.047, q = 0.116) and the ModDisc group (34.300 ± 0.165 cm; p = 0.040, q = 0.116); these differences did not persist after BH adjustment. Infants in the HighMod group (34.000 ± 0.162 cm) had a smaller head circumference than those in the Abstain group (34.500 ± 0.093 cm; p = 0.014, q = 0.070); this difference was marginal after BH adjustment.

Bayley MDI at 6 Months Analysis of 6-month MDI scores showed a linear association with alcohol trajectory group (β = -3.248; SE = 1.487; p = 0.029). There was no association with MVM status.

Post-hoc analysis of the overall alcohol trajectory effect, averaging across MVM supplementation statuses, indicated that infants in the VHigh group (83.5 ± 1.430) had lower 6-month MDI scores compared to infants in the Abstain group (90.7 ± 0.474; p < 0.0001; q < 0.0001), the Low group (89.9 ± 1.090; p = 0.0004; q = 0.0009), the ModDisc group (92.1 ± 0.938; p < 0.0001; q < 0.0001), and the HighMod group (90.0 ± 0.950; p = 0.0001; q = 0.0004) (Figure 3.18).

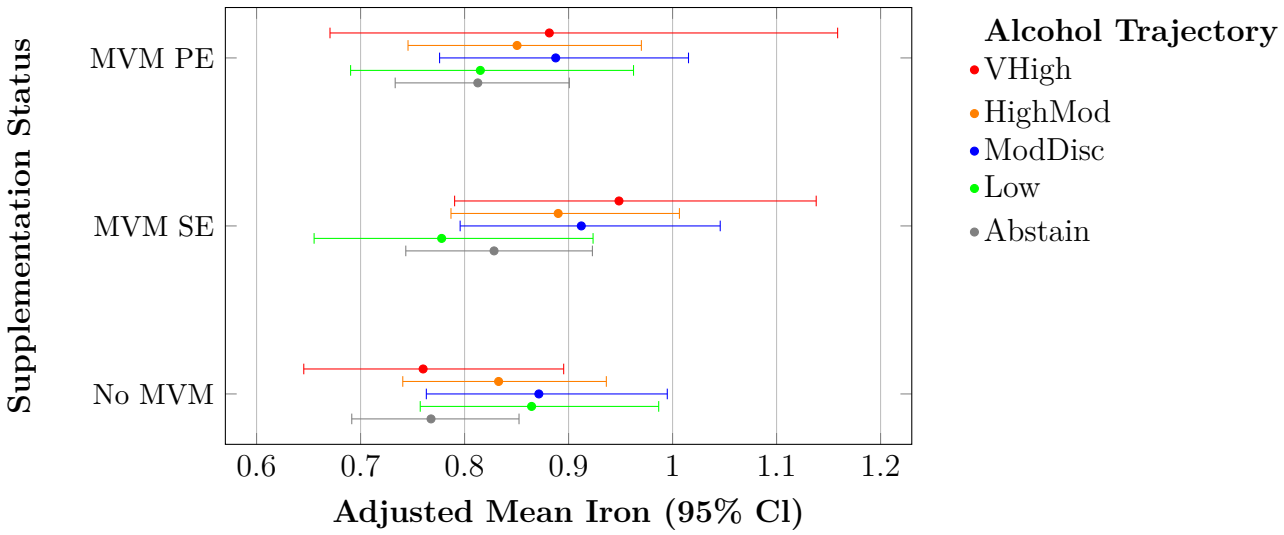


Figure 3.15: Forest plot of adjusted mean plasma iron (µg/mL) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups within each maternal alcohol consumption trajectory. Estimates are from a GLMM.

In the Abstain group, infants whose mothers initiated MVM SE (91.9 ± 0.851) had higher 6-month MDI scores than those in the No MVM group (89.8 ± 0.697; p = 0.048), a difference not present after BH adjustment (q = 0.143) (Figure 3.19). In the ModDisc group, infants whose mothers initiated MVM SE (94.1 ± 1.740) showed higher 6-month MDI scores compared to the No MVM group (89.4 ± 1.480; p = 0.038), though this comparison was not significant after BH adjustment (q = 0.113).

Within the No MVM group, 6-month MDI scores for infants in the VHigh group (85.9 ± 2.040) trended lower than scores for infants in the Abstain group (89.8 ± 0.697; p = 0.063) and were lower than scores for the Low group (91.1 ± 1.530; p = 0.040) (Figure 3.19). After BH adjustment, neither difference was significant.

Within the MVM SE group, infants in the VHigh group (80.5 ± 2.810) had lower 6-month MDI scores compared to infants in the Abstain group (91.9 ± 0.851; p < 0.001; q = 0.001), the Low group (90.1 ± 2.130; p = 0.006, q = 0.014), the ModDisc group (94.1 ± 1.740; p < 0.001; q < 0.001), and the HighMod group (89.6 ± 1.660; p = 0.005; q = 0.014) (Figure 3.19). These differences remained significant after adjustment. In contrast,

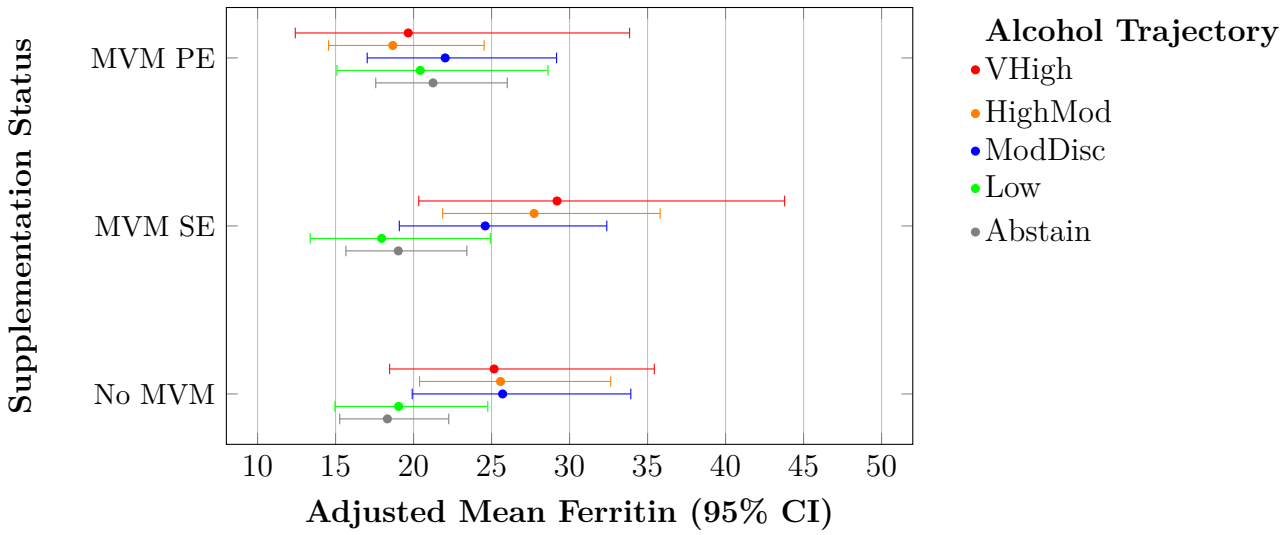


Figure 3.16: Forest plot of adjusted mean plasma ferritin (ng/mL) levels with 95% confidence intervals. Values are displayed across MVM supplementation groups within each maternal alcohol consumption trajectory. Estimates are from a GLMM.

while the ModDisc group was also marginally higher than the HighMod group (p = 0.057; q = 0.114), this difference did not remain significant after adjustment. Within the MVM PE group, infants in the VHigh group (84.2 ± 2.440) had lower 6-month MDI scores compared to infants in the Abstain group (90.4 ± 0.790; p = 0.015; q = 0.050), the ModDisc group (92.8 ± 1.680; p = 0.004; q = 0.037), and the HighMod group (91.7 ± 1.830; p = 0.014; q = 0.050).

Bayley PDI at 6 Months Analysis of 6-month PDI scores using the GLM indicated a main effect for the cubic component of the alcohol trajectory group (β = -6.184; SE = 2.240; p = 0.006). No main effect for MVM supplementation status occurred.

Post-hoc analysis of the overall alcohol trajectory effect, averaging across MVM supplementation statuses, showed infants in the VHigh group had lower 6-month PDI scores (79.9 ± 2.140) compared to infants in the Abstain group (89.2 ± 0.774; p < 0.001; q = 0.0003), the Low group (87.7 ± 1.830; p = 0.0055; q = 0.0138), the ModDisc group (90.6 ± 1.400; p < 0.0001; q = 0.0003), and the HighMod group (89.3 ± 1.410; p = 0.0002; q = 0.0007)

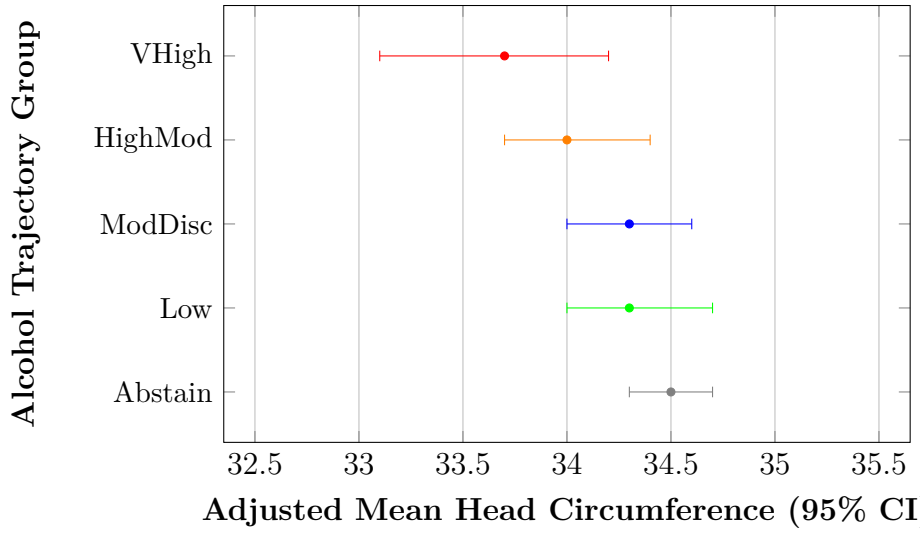


Figure 3.17: Forest plot shows adjusted mean head circumference size (cm), averaged across MVM supplementation status, with 95% confidence intervals for each alcohol trajectory. Estimates derive from a GLM.

(Figure 3.18). All these differences remained after BH adjustment.

Comparisons of MVM supplementation status within each alcohol trajectory group indicated that in the ModDisc group, infants whose mothers initiated MVM SE (94.0 ± 2.480) had higher 6-month PDI scores than those in the No MVM group (86.7 ± 2.400; p = 0.0365; q = 0.1094); this difference did not persist after BH adjustment (Figure 3.20). No other differences in PDI scores based on MVM status occurred within the other alcohol trajectory groups.

Within the No MVM group, infants in the HighMod group (91.5 ± 2.080) had higher 6-month PDI scores than those in the Low group (84.6 ± 2.460; p = 0.0306; q = 0.1528) (Figure 3.20). Infants in the VHigh group (83.0 ± 2.900) had lower scores than those in the HighMod group (p = 0.0174; q = 0.1528). A trend for lower scores was present for the VHigh group compared to the Abstain group (88.7 ± 1.190; p = 0.0707; q = 0.2356). These differences failed to reach significance after BH adjustment.

Within the MVM SE group, infants in the VHigh group (76.4 ± 3.770) had lower 6-month PDI scores compared to infants in the Abstain group (90.7 ± 1.440; p = 0.0005; q = 0.0024), the Low group (89.7 ± 3.580; p = 0.0107; q = 0.0355), the ModDisc group (94.0

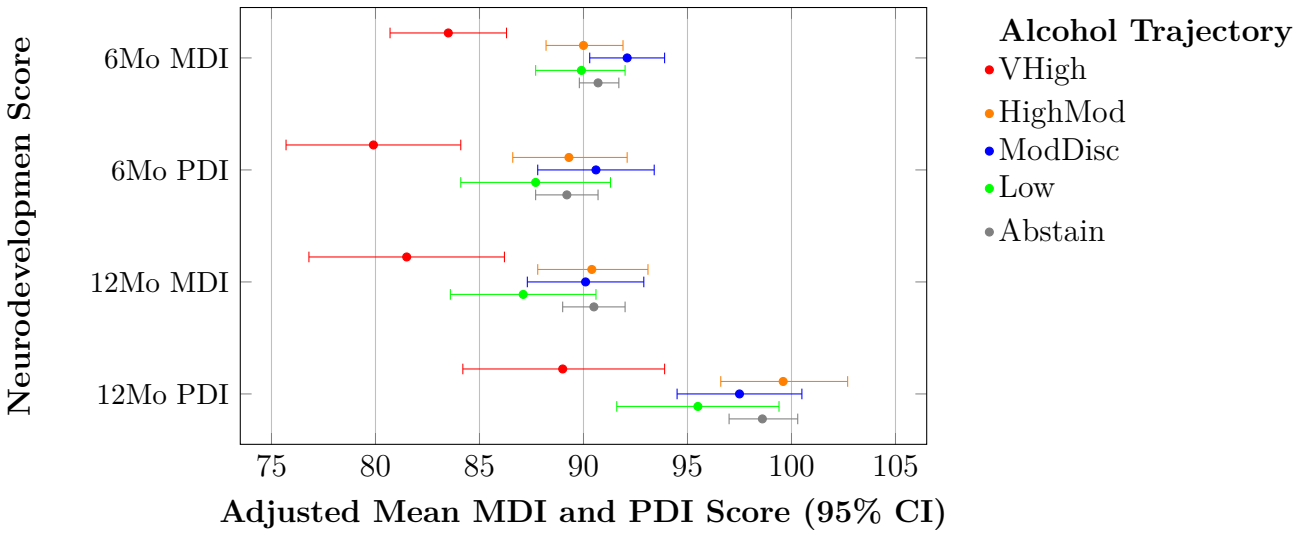


Figure 3.18: Forest plot shows adjusted mean MDI and PDI scores at 6 months (6Mo) and 12 months (12Mo), averaged across MVM supplementation status, with 95% confidence intervals for each alcohol trajectory. Estimates derive from a GLM.

± 2.480; p < 0.0001; q = 0.0010), and the HighMod group (86.9 ± 2.250; p = 0.0163; q = 0.0409) (Figure 3.20). All these comparisons remained after BH adjustment. Infants in the ModDisc group had higher scores than those in the HighMod group (p = 0.0329; q = 0.0658); this difference was not present after BH adjustment.

Within the MVM PE group, infants in the VHigh group (80.2 ± 4.270) had lower 6-month PDI scores compared to those in the ModDisc group (91.1 ± 2.450; p = 0.0289; q = 0.2574) (Figure 3.20). Trends for lower scores in the VHigh group were present when compared to the Abstain group (88.2 ± 1.260; p = 0.0772; q = 0.2574) and the HighMod group (89.6 ± 2.730; p = 0.0643; q = 0.2574). Following BH adjustment, these differences within the MVM PE group were no longer significant.

Bayley MDI at 12 Months Analysis of 12-month MDI scores showed a linear association with alcohol trajectory group (β = -4.684; SE = 2.211; p = 0.034). No main effect for MVM supplementation status was observed, and no interaction between alcohol trajectory group and MVM supplementation status was detected.

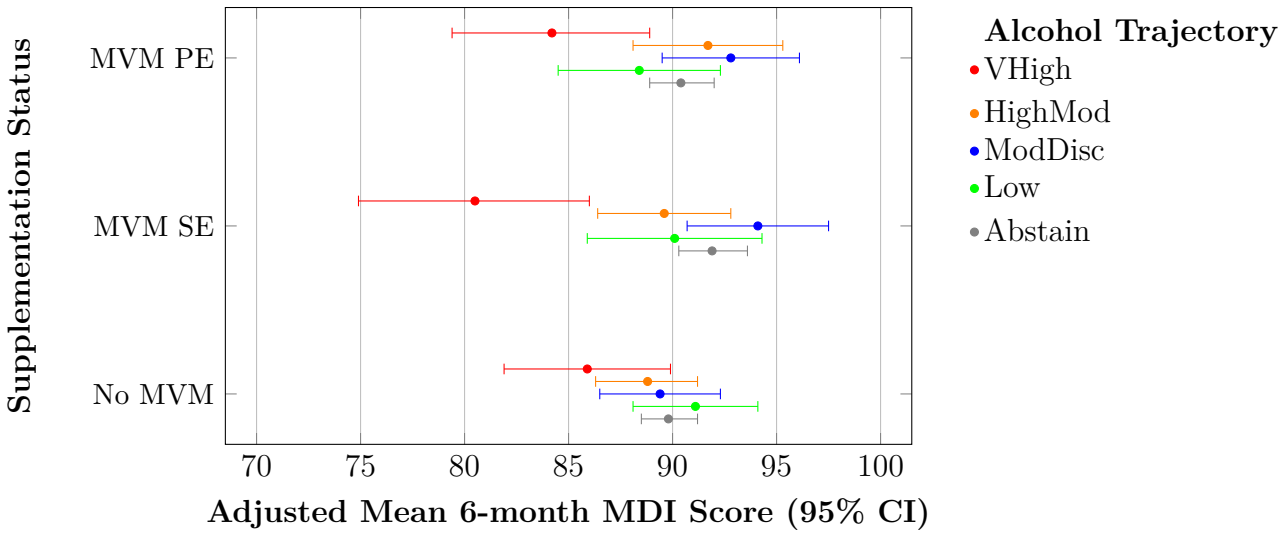


Figure 3.19: Forest plot shows adjusted mean MDI scores at 6 months with 95% confidence intervals for each alcohol trajectory. Estimates derive from a GLM.

Post-hoc analysis of the overall alcohol trajectory effect, averaging across MVM supplementation statuses, showed that infants in the VHigh group (81.5 ± 2.410) had lower 12-month MDI scores compared to infants in the Abstain group (90.5 ± 0.774; p = 0.0005; q = 0.0046), the ModDisc group (90.1 ± 1.420; p = 0.0019; q = 0.0064), and the HighMod group (90.4 ± 1.350; p = 0.0010; q = 0.0049) (Figure 3.18). The difference between the VHigh group and the Low group (87.1 ± 1.790; p = 0.0608; q = 0.1520) did not persist after BH adjustment.

Bayley PDI at 12 Months For 12-month PDI scores, the GLM analysis indicated no main effect for alcohol trajectory group. A main effect for MVM supplementation status was found, where infants whose mothers were in the MVM SE group had lower scores compared to infants whose mothers were in the No MVM group (β = -3.901; SE = 1.825; p = 0.033).

Post-hoc analysis of the overall MVM supplementation effect showed that infants in the MVM SE group (93.6 ± 1.47) had lower 12-month PDI scores than infants in the No MVM group (97.5 ± 1.12; p = 0.033); this difference was not significant after BH adjustment (q = 0.098) (Figure 3.21).

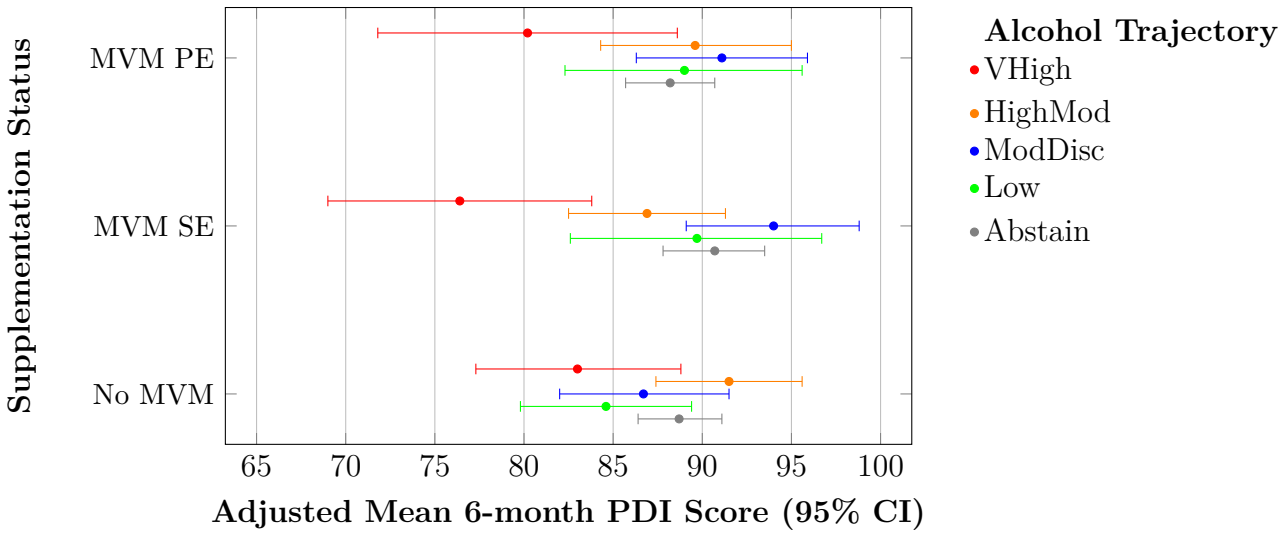


Figure 3.20: Forest plot shows adjusted mean PDI scores at 6 months with 95% confidence intervals for each alcohol trajectory. Estimates derive from a GLM.

Exploring comparisons of MVM supplementation status within each alcohol trajectory group showed that in the VHigh alcohol trajectory group, infants whose mothers were in the MVM SE group (82.1 ± 4.75) had lower 12-month PDI scores compared to infants whose mothers were in the No MVM group (95.2 ± 3.09; p = 0.0201); this difference was marginal after BH adjustment (q = 0.0603) (Figure 3.21). No other significant differences in 12-month PDI scores based on MVM status were observed within the other alcohol trajectory groups.

Among participants in the No MVM group, 12-month PDI scores did not differ significantly across alcohol trajectory groups.

Among participants in the MVM SE group, infants in the VHigh group (82.1 ± 4.75) had lower 12-month PDI scores compared to infants in the Abstain group (97.1 ± 1.49; p = 0.0027; q = 0.0136), the ModDisc group (99.5 ± 2.76; p = 0.0013; q = 0.0131), and the HighMod group (96.7 ± 2.53; p = 0.0061; q = 0.0202) (Figure 3.21). A trend towards lower scores was observed for the VHigh group compared to the Low group (92.8 ± 3.70; p = 0.0763; q = 0.1908), which did not persist after BH adjustment.

Among participants in the MVM PE group, infants in the VHigh group (89.8 ± 4.78) had lower 12-month PDI scores compared to infants in the Abstain group (99.7 ± 1.34; p =

0.0480; q = 0.2399) and the HighMod group (102.2 ± 2.96; p = 0.0293; q = 0.2399); these differences did not remain significant after BH adjustment (Figure 3.21).

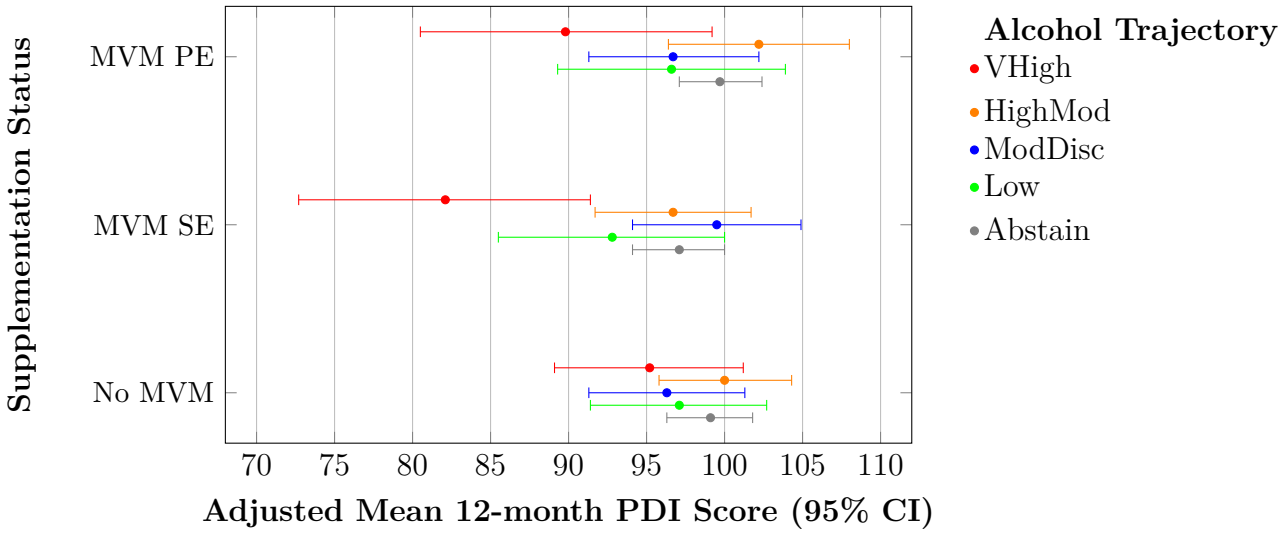


Figure 3.21: Forest plot shows adjusted mean PDI scores at 12 months with 95% confidence intervals for each alcohol trajectory. Estimates derive from a GLM.

3.4 Discussion

This investigation characterized distinct prenatal alcohol consumption trajectories and found these patterns correlated with unique maternal biochemical profiles. Trajectories involving higher alcohol intake frequently showed greater physiological disruption, including markers of oxidative stress, inflammation, and altered nutrient status. MVM supplementation attenuated some of these alcohol-related biochemical alterations.

3.4.1 Prenatal Alcohol Exposure, Oxidative Stress, and the Modulatory Role of Multivitamin/Multimineral Supplementation

A primary mechanism by which PAE is thought to exert its damaging effects is the induction of oxidative stress. Oxidative stress is a state characterized by an imbalance between ROS

production and the body's ability to detoxify these reactive intermediates or repair the resulting damage[65].

TBARS: A Marker of Lipid Peroxidation

Our study found that women with VHigh alcohol intake during pregnancy exhibited significantly elevated TBARS levels, reflecting increased oxidative lipid damage compared to other alcohol intake trajectories. This observation aligns with previous human studies demonstrating that chronic alcohol consumption heightens markers of lipid peroxidation, including (MDA) and lipid hydroperoxides[66,67]. A dose-response relationship, where oxidative damage is substantially higher among individuals with greater alcohol consumption, is also documented in human populations[68].

Antioxidant Defense System Response to PAE

The body employs a complex antioxidant defense system to counteract ROS. This system includes enzymatic components such as SOD, GRed, and GPx, alongside non-enzymatic antioxidants whose collective capacity can be assessed by measures like TRAP[69].

SOD is a principal enzymatic antioxidant that converts superoxide radicals into hydrogen peroxide, a molecule subsequently detoxified by enzymes like GPx[70,71]. In our study, the VHigh alcohol intake category within the No MVM group exhibited the lowest average erythrocyte SOD activity, though differences between alcohol trajectory groups did not reach statistical significance. This observation aligns with previous research reporting decreased erythrocyte SOD activity in individuals with alcohol dependence and in animal models of alcohol exposure[70]. Alcohol consumption can reduce levels of zinc, an important cofactor for SOD, and ethanol metabolites may directly inactivate SOD enzymes, potentially explaining such decreases[70].

GRed maintains cellular redox balance by catalyzing the reduction of GSSG to its active form, GSH, using NADPH as an electron donor[72]. Our analyses showed a quadratic

association between increased alcohol intake and decreased GRed activity. Participants with VHigh alcohol consumption showed numerically lower, though not statistically significant, GRed activity across all MVM supplementation categories compared with the Abstain group. A clear gradient was not apparent across other alcohol trajectory groups. These findings are consistent with a varied body of literature, where alcohol exposure has been found to either upregulate[73] or downregulate GRed activity[74,75].

Erythrocyte GPx contributes to cellular antioxidant defense by catalyzing the reduction of hydrogen peroxide and lipid hydroperoxides to water and their corresponding alcohols, utilizing GSH as a substrate[76]. In our study, GPx activity did not differ significantly across alcohol trajectory groups or MVM statuses. The literature concerning erythrocyte GPx activity in the context of alcohol consumption presents mixed findings. One human study reported downregulated GPx activity among alcohol consumers[77]. Conversely, a meta-analysis found decreased erythrocyte GPx activity in patients with alcohol dependence but also noted that overall changes in GPx activity might be nuanced, potentially varying by tissue type or sex, or influenced by factors such as acetaldehyde-mediated inhibition[70].

The TRAP assay evaluates the overall antioxidant capacity of plasma, reflecting the synergistic effects of multiple antioxidant components[78]. Key contributors to plasma TRAP include urate, plasma proteins, ascorbate, and vitamin E[79]. Our study found that TRAP levels increased in a manner described by linear and cubic relationships with the alcohol intake trajectories.

The Nrf2 pathway, a primary regulator of the antioxidant defense system orchestrating the transcription of enzymes including SOD and GPx[80,81], offers a plausible mechanistic framework for interpreting some of our observations, particularly within the VHigh group. Research indicates that the Nrf2–antioxidant response element (ARE) pathway can exhibit a J-shaped dose-response, with robust protective activation at higher levels of oxidative stress[82]. Furthermore, repeated xenobiotic exposures, while potentially dampening initial Nrf2 accumulation, may lead to enhanced induction of downstream targets, suggesting that

cells can become primed for a more vigorous antioxidant response upon subsequent challenges[83]. It is conceivable that under conditions of VHigh alcohol intake, persistent oxidative stress could similarly prime elements of the antioxidant defense system.

MVM Supplementation and Mitigation of Oxidative Stress

The data indicated MVM supplementation played a modulatory role in PAE-induced oxidative stress. The study showed that MVM supplementation, initiated either at or prior to enrollment, attenuated oxidative lipid damage, reflected by lower TBARS, in the VHigh group. This observation corresponds with experimental animal models where antioxidant supplementation, such as with quercetin, reduced TBARS levels in ethanol-exposed rats. Quercetin, a natural antioxidant, was shown to decrease plasma TBARS and protein carbonyls, and increase GSH levels, providing protection against ethanol-induced oxidative damage[84].

Previous research indicates supplementation can restore erythrocyte SOD activity[85,86]; for instance, glycine supplementation restored SOD activity in rats with alcohol-induced liver injury[85], and various antioxidants restored SOD activity in patients with alcoholic cirrhosis[86]. In our subjects, the VHigh group receiving MVM supplementation showed increased SOD activity relative to their unsupplemented counterparts; participants in the MVM PE subgroup demonstrated higher SOD activity than participants in other alcohol intake groups. A comparable trend was observed among those who started MVM at enrollment. This restoration may relate in part to improved status of zinc and copper, cofactors for SOD[87], which alcohol consumption can deplete, thereby compromising enzymatic oxidative defense systems. The potential priming of the Nrf2 pathway in the VHigh group, coupled with the provision of necessary cofactors and antioxidants from MVM, might explain the heightened SOD and TRAP activity observed in supplemented high-exposure individuals.

MVM supplementation was associated with higher GRed activity across all alcohol intake groups. The group with supplementation initiated before enrollment demonstrated the

greatest increase in enzyme activity compared to the unsupplemented group. This observation may relate to erythrocyte GRed dependency on flavin adenine dinucleotide (FAD), a cofactor derived from riboflavin[88]. Erythrocyte GRed activity is used as a biomarker of riboflavin nutritional status[88,89], and riboflavin deficits often manifest as reduced GRed activity[90]. Women during pregnancy are at heightened risk of riboflavin deficiency[91]. The observed enhancement in GRed activity with longer-term MVM supplementation may reflect improved B vitamin status, a conclusion supported by studies showing that multivitamin interventions bolster B vitamin levels in women[92].

Despite selenium being a catalytic cofactor of GPx and its inclusion in MVM supplements, our results did not show any modulation of GPx by either alcohol exposure patterns or MVM supplementation, contrasting with studies showing selenium supplementation can increase GPx activity in plasma and erythrocytes[93,94]. Similarly, MVM supplementation did not significantly alter TRAP levels overall, although within the MVM PE group, participants in the VHigh alcohol group exhibited elevated TRAP levels. An animal study established that ethanol ingestion perturbs the plasma antioxidant system in a dose- and time-dependent manner; specifically, antioxidant markers were observed to increase within 2–4 hours following ethanol exposure before subsequently dissipating[95]. This temporal pattern suggests that ethanol consumption may transiently boost antioxidant activity which may not have been fully captured in our study design.

3.4.2 Prenatal Alcohol Exposure, Inflammation, the Acute Phase Response, and Nutrient Homeostasis

Beyond directly impacting redox balance, PAE can initiate systemic inflammatory responses, including the APR, which may exacerbate nutrient dysregulation and contribute to adverse developmental outcomes.

A central hypothesis posits that PAE induces a conditional nutrient deficiency, reducing fetal nutrient availability even if a mother has adequate nutrient intake[14]. A key mechanism

involves an alcohol-triggered APR, characterized by cytokine-mediated upregulation of hepatic MT synthesis[14,19,96]. Increased MT expression sequesters zinc in maternal liver tissue, diminishing placental zinc transfer during critical developmental windows[14,97,98]. Rodent studies confirm that an alcohol-induced APR correlates with reduced fetal zinc uptake and increased teratogenicity; zinc supplementation can ameliorate these effects[14,99]. MT knockout models further support this by abolishing alcohol-related zinc dysregulation and fetal deficits[19].

APR activation also alters maternal iron and copper homeostasis[96]. Plasma iron concentrations often decline due to hepcidin-mediated sequestration, a hormone regulating iron flow into plasma[100]. Conversely, CP-driven elevations in plasma copper can occur; CP, a copper-binding acute phase protein, also functions as a free radical scavenger, potentially mitigating oxidative tissue injury[101,102].

hsCRP: A Marker of Systemic Inflammation

hsCRP, a sensitive marker of systemic inflammation, mediates the APR[103]. Our observation that hsCRP elevation corresponded with higher alcohol intake aligns with alcohol's capacity to trigger inflammation, a process potentially mediated by oxidative stress[104]. Unsupplemented participants in the VHigh alcohol trajectory exhibited significantly higher hsCRP than lower-exposure groups; this finding is congruent with prior reports that chronic alcohol provokes an APR[103,104].

Importantly, MVM supplementation appeared to mitigate this effect. Among VHigh participants initiating MVM at enrollment, hsCRP levels became comparable to those in lower-exposure trajectories. For pre-enrollment MVM users in the VHigh group, hsCRP levels were lower than those of abstainers. This reduction may reflect an overcompensation of antioxidant defenses. Elevated SOD and TRAP activity in supplemented high-intake groups, as discussed earlier, likely attenuated oxidative stress, which could indirectly suppress APR activation, even with continued alcohol exposure[103].

Impact on Essential Minerals: Zinc and Copper

Zinc supports fetal neurodevelopment; deficiencies, even marginal ones, can affect neurogenesis and neuronal apoptosis, leading to adverse outcomes[105]. Our findings indicated that unsupplemented participants in the VHigh alcohol trajectory exhibited lower plasma zinc levels than other groups. This observation is consistent with prior work establishing that alcohol disrupts zinc homeostasis by reducing intestinal absorption[15] and potentially increasing excretion, partly because key enzymes in alcohol metabolism, such as ADH, are zinc-dependent metalloenzymes[106].

Copper is vital for neurodevelopment, and its deficiency, reported among heavy alcohol consumers, may contribute to alcohol-related central nervous system abnormalities[107]. Our study identified an inverse quadratic association between PAE trajectories and maternal plasma copper concentrations, most apparent among unsupplemented participants. The VHigh alcohol trajectory group displayed lower copper levels compared to low or moderate-exposure groups. This pattern parallels findings from rodent studies where maternal ethanol intake, particularly with low dietary copper, led to hepatic copper depletion in both dams and pups, potentially through mechanisms such as enhanced biliary excretion[23,25].

MVM supplementation showed a capacity to affect these mineral balances. Initiating MVM supplementation at enrollment (MVM SE) appeared to counteract alcohol-related zinc deficits in both the HighMod and VHigh groups. Supplementation prior to enrollment (MVM PE) showed a comparable, though not statistically distinct, effect. These results are consonant with animal models where zinc supplementation during ethanol exposure reduced teratogenicity and improved fetal viability[108], restored aspects of immune function[109], and inhibited alcohol-induced apoptosis, possibly by mitigating oxidative stress[110]. For copper, initiating MVM supplementation at enrollment also appeared to lessen alcohol-related copper depletion. The VHigh and HighMod groups using MVM from enrollment maintained copper levels comparable to lower-exposure trajectories. This observation is supported by evidence that dietary copper supplementation can mitigate alcohol-induced oxidative stress,

potentially by protecting gut barrier integrity[22]. Pre-enrollment MVM use improved copper status in the HighMod group. The VHigh subgroup with pre-enrollment MVM use, however, retained lower copper concentrations relative to other supplemented trajectories. This persistent decrease might reflect limitations of the supplementation classification or other unclarified underlying mechanisms.

Iron Homeostasis: A Complex Interaction

Our study observed decreasing plasma iron concentrations with higher alcohol intake, although differences between trajectory groups and supplementation statuses were not statistically significant. This finding may reflect alcohol's biphasic effects on iron metabolism[28]. Moderate alcohol intake can reduce iron deficiency risk in non-pregnant populations, partly by suppressing hepcidin and thereby augmenting intestinal iron absorption[111]. Conversely, heavy, sustained alcohol consumption, such as that seen in our VHigh trajectory, may disrupt iron homeostasis. For instance, alcohol-induced oxidative stress can downregulate hepcidin expression, potentially leading to increased intestinal iron absorption and altered iron storage[33]. Clinically, maternal binge drinking is associated with a tripled risk of infant iron deficiency anemia, possibly through impaired fetal iron accumulation[28]. Developmental iron deficiency, even without anemia, adversely affects neurobehavioral outcomes, linked to vulnerabilities in specific brain regions and impaired myelination[112,113]. Rodent models indicate that iron repletion or maternal supplementation can partially ameliorate some effects of iron deficiency or PAE on fetal growth and neurochemistry[114,115]. Our findings suggest MVM supplementation during pregnancy did not directly modulate maternal iron status as measured by plasma iron. Alcohol's threshold-dependent effects on absorption pathways may obscure more subtle nutrient interactions. We also did not find that MVM supplementation affected plasma TfR values, another indicator of iron status.

However, ferritin levels increased linearly with alcohol intake severity; participants in ModDisc, HighMod, and VHigh trajectories exhibited elevated concentrations compared

to the Abstain and Low groups in both unsupplemented and MVM SE cohorts. These observations align with studies connecting elevated serum ferritin in alcohol consumers with hepatocellular injury rather than primarily with systemic iron overload[116,117]. Alcohol itself may also induce ferritin synthesis[118]. Ferritin's role as an acute phase reactant, where stimuli such as cytokines increase its synthesis or secretion, presents another mechanism for its elevation[119].

MVM supplementation initiated prior to enrollment attenuated alcohol trajectory-associated differences in ferritin. This suggests that ongoing micronutrient support may reduce alcohol-related hepatic damage. Antioxidants in MVMs, such as vitamins C and E, may mitigate oxidative stress from ethanol metabolism, potentially limiting hepatocellular injury and subsequent ferritin leakage into circulation[120,121]. Specifically, antioxidants have been shown to counteract alcohol's impact on hepatic hepcidin expression by reducing oxidative stress[120].

3.4.3 Impact of Prenatal Alcohol Exposure Trajectories and MVM Supplementation on Infant Outcomes

The preceding discussion detailed the relationship between patterns of prenatal alcohol consumption and maternal biochemical profiles, and the modulatory effects of MVM supplementation on some of these maternal markers. This section evaluates the impact of these PAE trajectories and maternal MVM use on infant physical growth and neurodevelopment.

This study investigated associations between these longitudinally defined PAE patterns, maternal MVM supplementation, and infant outcomes, such as physical growth at birth and neurodevelopment at 6 and 12 months. A key finding was a dose-dependent negative association between higher PAE trajectories and several adverse infant outcomes, especially reduced head circumference and lower neurodevelopmental scores. Maternal MVM supplementation conferred some benefits to maternal biochemical markers, as previously discussed. Nutrient supplementation did not provide extensive protective effects against these PAE-induced impairments in infant physical growth or neurodevelopment.

Physical Growth Parameters

Infant birthweight and length at birth were not significantly associated with maternal alcohol trajectory group or MVM supplementation status. This outcome differs from some reports where heavy PAE is linked to reduced birthweight or length, which are recognized features in FASD diagnostic criteria[122–124]. Bandoli et al. (2019), employing trajectory modeling within a similar CIFASD cohort, reported associations between the highest alcohol consumption trajectory and reduced birthweight and length percentiles[37].

Our study found a negative linear association between higher PAE trajectories and smaller infant head circumference at birth, aligning with established consequences of PAE[123,124]. Reduced head circumference is a key diagnostic criterion for FAS and reflects impaired brain growth[122]. The developing brain is particularly vulnerable to ethanol's teratogenic effects, which can interfere with cell proliferation, migration, and differentiation. This finding for head circumference contrasts with the study by Bandoli et al. (2019), which did not detect a significant association between their PAE trajectories and head circumference after adjustments[37]. In the current investigation, maternal MVM supplementation did not modify the association between PAE trajectory and infant head circumference. This lack of modification by MVM is of interest, as other research indicates that different nutritional factors, such as maternal gestational weight gain or specific dietary components like choline and iron, can alter the impact of PAE on infant head circumference[123].

Neurodevelopmental Scores

Higher PAE trajectories consistently predicted adverse infant neurodevelopment. A linear negative association appeared between alcohol trajectory group and MDI scores at both 6 and 12 months (Figure 3.18). Infants in the VHigh group demonstrated the lowest MDI scores compared to all other trajectory groups at 6 months, and relative to the Abstain, ModDisc, and HighMod groups at 12 months. These outcomes align with extensive research documenting the neurotoxic effects of alcohol on the developing brain, which can lead to cog-

nitive and learning disabilities characteristic of FASD[122]. The MDI assesses early cognitive and language abilities, domains previously shown to be vulnerable to PAE[37,125].

For 6-month PDI scores, which evaluate motor skills, infants in the VHigh trajectory group had substantially lower scores than infants in all other trajectory groups (Figure 3.18). This finding suggests that early motor development is also susceptible to dose-dependent effects of PAE.

Maternal MVM supplementation did not demonstrate a clear, overarching protective effect against these neurodevelopmental consequences of PAE. No main effect of MVM status occurred for 6-month or 12-month MDI scores, nor for 6-month PDI scores. Some isolated within-group comparisons hinted at potential slight benefits of MVM SE for 6-month MDI scores in the Abstain group and the ModDisc group (Figure 3.19) before BH correction. Many women in the ModDisc group reduced their alcohol intake after pregnancy recognition. This observation, coupled with the findings from Bandoli et al. (2019) in a similar cohort suggesting that prolonged exposure, even at modest amounts, can be detrimental, points to a potential nuanced role for nutrient supplementation[37]. If maternal behavior changes to reduce or cease alcohol consumption, MVM supplementation initiated around pregnancy recognition, as in the MVM SE group, might offer some support against the effects of earlier gestational exposure. This aligns with the call from Bandoli et al. (2019) for interventions even after the first trimester[37]. Coles et al. (2015) also reported a protective effect of MVM on cognitive scores in this Ukrainian cohort[125]. For infants in the VHigh trajectory in the current study, MDI and PDI scores at 6 months remained significantly lower than other trajectory groups, irrespective of maternal MVM supplementation (Figures 3.19, 3.20). This pattern suggests either the level or length of nutritional support from the MVM used in this study may be insufficient to counteract the severe neurodevelopmental impact of very high, sustained PAE or that nutritional supplementation may not be a viable intervention for this high alcohol-exposed group.

The results for 12-month PDI scores presented a more intricate pattern. No main effect

for alcohol trajectory group was detected. An unexpected main effect for MVM supplementation status emerged, where infants whose mothers were in the MVM SE group had lower 12-month PDI scores compared to infants whose mothers were in the No MVM group; this overall effect did not persist after BH adjustment. Examination within alcohol trajectory groups (Figure 3.21) showed this negative association was particularly apparent in the VHigh alcohol trajectory group, where MVM SE was linked to lower 12-month PDI scores compared to No MVM. Within both the MVM SE and MVM PE groups, infants from the VHigh trajectory had lower 12-month PDI scores than those from several lower exposure trajectories. This counterintuitive result for MVM SE and 12-month PDI requires cautious interpretation. It could represent an anomalous finding, reflect unmeasured confounding factors, or indicate complex interactions between specific nutrients, high alcohol exposure, and particular developmental pathways not yet fully understood. The finding underscores that MVM supplementation, as provided, did not confer a benefit for 12-month motor outcomes, particularly with very high PAE. The complexity of nutrient interactions and their impact on development, especially in unique populations such as the Ukrainian cohort studied (which, for example, does not have folic acid enrichment in food products), has been noted previously[126,127]. Moreover, early infant assessments like the BSID-II may not capture the full spectrum of PAE effects[122,125].

3.4.4 Strengths and Limitations

This investigation has several strengths. The application of GMM to delineate longitudinal PAE trajectories provided a more detailed understanding of exposure patterns compared to binary alcohol use classifications. The study associated these PAE trajectories with a panel of maternal biochemical markers, including those for oxidative stress, antioxidant defense, and micronutrient status. This investigation linked these detailed PAE patterns directly to maternal nutritional physiology, a novel aspect. This approach expands on prior work within the CIFASD cohort that used trajectory modeling for PAE but focused mainly on

infant outcomes without the same characterization of maternal biochemical status[37].

The examination of MVM supplementation effects across a spectrum of prenatal alcohol consumption, including a comparison group of women who abstained, is another strength. This design permitted assessment of MVM impact on maternal biochemical profiles and infant development across varying PAE levels. Previous studies from this cohort investigated micronutrient supplementation[125–127]. The current work advances this by examining the interaction between longitudinally defined PAE, maternal biochemical responses, and MVM intervention. Analyzing repeated maternal biomarker measurements with GLMMs facilitated the inclusion of participants with varying numbers of study visits. This statistical approach maximized data use and appropriately accounted for within-subject correlations. Alcohol reporting in the Ukrainian research setting may be less subject to stigma than in other regions, potentially benefiting reporting accuracy.

Several limitations merit consideration. Results may have limited generalizability. The Western Ukrainian cohort has distinct characteristics, such as no folic acid food fortification, potential Chernobyl impacts[126,127]. These factors could influence baseline nutritional status and MVM supplementation responsiveness. The observed effects may not apply directly to populations with different diets, genetics, or healthcare contexts[125,128]. The sample, primarily a high-risk population for PAE, may affect the identified consumption trajectories compared to lower-risk or general population samples[37].

Maternal self-report of alcohol consumption, despite standardized TLFB methodology, is a potential bias source. Systematic underreporting or recall inaccuracies linked to trajectory group membership could influence estimated associations[37]. Residual confounding from unmeasured variables (e.g., detailed maternal diet beyond supplements, paternal factors, genetic polymorphisms influencing alcohol or nutrient metabolism, environmental exposures) remains possible, despite statistical control of many potential confounders[37,128]. The lack of detailed dietary intake data[126] complicates assessing how pre-existing dietary patterns might have interacted with the MVM intervention.

The MVM intervention design, where a subset received choline, was simplified for the maternal biomarker analyses presented, as choline showed no additional effect on these specific outcomes. This simplification means the unique contribution of choline, or its interactions with MVM components for other outcomes, was not fully elucidated here[126,127]. Selecting the four-class GMM solution involved statistical fit indices and theoretical considerations, but determining the optimal number of latent trajectories inherently includes subjectivity.

The BSID-II scales, administered early in development, may not capture the full spectrum or long-term manifestations of PAE-related neurodevelopmental effects[37,128]. Some cognitive domains, such as verbal function, were not assessed in this cohort due to the research setting[128]. Nutrient metabolism complexity and interactions suggest a standard MVM formulation might not be universally optimal across different PAE levels or for all individuals[126]. The unexpected finding of lower 12-month PDI scores in the MVM SE group, particularly within the VHigh PAE trajectory, illustrates this complexity. Caution is needed when drawing definitive conclusions about MVM efficacy for all outcomes, especially with very high alcohol exposure. Future investigations could explore these relationships in diverse populations, incorporate more detailed dietary assessments, and conduct longer-term follow-up of developmental outcomes.

3.5 Conclusion

This investigation identified distinct prenatal alcohol consumption trajectories associated with altered maternal oxidative stress, antioxidant defense, inflammation, and nutrient status. MVM supplementation during pregnancy ameliorated some maternal biochemical disruptions, particularly in women with higher alcohol intake. Yet higher PAE trajectories were consistently associated with adverse infant outcomes, including reduced head circumference and poorer neurodevelopmental performance. Detailed trajectory modeling indicates that the MVM nutritional support provided in this study can modulate maternal physiological

responses to alcohol but may not be sufficient to counteract the detrimental effects of PAE on the developing fetus. Abstinence from alcohol during pregnancy remains the most effective approach to prevent alcohol-related fetal harm; future research should explore more targeted or comprehensive intervention strategies for mitigating risks in exposed pregnancies.

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Bibliography

[1] Svetlana Popova. "Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis". en. In: *The Lancet Global Health* 5.3 (2017), p. 10. DOI: 10.1016/S2214-109X(17)30021-9.

[2] Shannon Lange et al. "Global prevalence of fetal alcohol spectrum disorder among children and youth: a systematic review and meta-analysis". en. In: *Obstetrical & Gynecological Survey* 73.4 (Apr. 2018), pp. 189–191. DOI: 10.1097/01.ogx.0000532194.88210.00.

[3] Svetlana Popova et al. "Prevalence of fetal alcohol spectrum disorder among special subpopulations: a systematic review and meta-analysis". en. In: *Addiction* 114.7 (July 2019), pp. 1150–1172. DOI: 10.1111/add.14598.

[4] Philip A. May et al. "The prevalence of fetal alcohol spectrum disorders in rural communities in South Africa: a third regional sample of child characteristics and maternal risk factors". en. In: *Alcoholism: Clinical and Experimental Research* 46.10 (Oct. 2022), pp. 1819–1836. DOI: 10.1111/acer.14922.

[5] Ira J. Chasnoff, Anne M. Wells, and Lauren King. "Misdiagnosis and missed diagnoses in foster and adopted children with prenatal alcohol exposure". en. In: *Pediatrics* 135.2 (Feb. 2015), pp. 264–270. DOI: 10.1542/peds.2014-2171.

[6] Ann P. Streissguth et al. “Risk Factors for Adverse Life Outcomes in Fetal Alcohol Syndrome and Fetal Alcohol Effects”. en-US. In: *Journal of Developmental & Behavioral Pediatrics* 25.4 (Aug. 2004), p. 228.

[7] Francesco Sessa et al. “Understanding the relationship between fetal alcohol spectrum disorder (FASD) and criminal justice: a systematic review”. en. In: *Health Care* 10.1 (Jan. 2022), p. 84. DOI: 10.3390/healthcare10010084.

[8] Mir M. Ali, Larry Burd, and Kristina D. West. *Estimating costs of health care for children with fetal alcohol spectrum disorder in a large health insurance claims database*. en. Aug. 2022. DOI: 10.21203/rs.3.rs-1930471/v1.

[9] Samir Zakhari. “Overview: How Is Alcohol Metabolized by the Body?” en. In: *Alcohol Res Health* 29.4 (2006), pp. 245–54.

[10] Arthur I. Cederbaum. “Alcohol Metabolism”. en. In: *Clinics in Liver Disease* 16.4 (Nov. 2012), pp. 667–685. DOI: 10.1016/j.cld.2012.08.002.

[11] Peter G. Wells et al. “Fetal oxidative stress mechanisms of neurodevelopmental deficits and exacerbation by ethanol and methamphetamine”. en. In: *im* 108.2 (June 2016). 00035, pp. 108–130. DOI: 10.1002/bdrc.21134.

[12] David González-Flores, Antonia Márquez, and Ilda Casimiro. “Oxidative Effects in Early Stages of Embryo Development Due to Alcohol Consumption”. en. In: *International Journal of Molecular Sciences* 25.7 (Jan. 2024), p. 4100. DOI: 10.3390/ijms25074100.

[13] Shama Bhatia et al. “Oxidative stress and DNA damage in the mechanism of fetal alcohol spectrum disorders”. en. In: *Birth Defects Research* 111.12 (July 2019), pp. 714–748. DOI: 10.1002/bdr2.1509.

[14] Carl L. Keen et al. “The plausibility of maternal nutritional status being a contributing factor to the risk for fetal alcohol spectrum disorders: the potential influence of zinc status as an example”. en. In: *BioFactors* 36.2 (Mar. 2010), pp. 125–135. DOI: 10.1002/biof.89.

[15] Dean L. Antonson and Jon A. Vanderhoof. “Effect of chronic ethanol ingestion on zinc absorption in rat small intestine”. en. In: *Digestive diseases and sciences* 28.7 (July 1983), pp. 604–608. DOI: 10.1007/BF01299920.

[16] Arthur Flynn et al. “Zinc status of pregnant alcoholic women: a determinant of fetal outcome”. en. In: *Lancet*. Originally published as Volume 1, Issue 8220 317.8220 (Mar. 1981), pp. 572–575. DOI: 10.1016/S0140-6736(81)92029-8.

[17] Brooke L. Summers, Allan M. Rofe, and Peter Coyle. “Dietary Zinc Supplementation Throughout Pregnancy Protects Against Fetal Dysmorphology and Improves Postnatal Survival After Prenatal Ethanol Exposure in Mice”. en. In: *Alcoholism: Clinical and Experimental Research* 33.4 (2009). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1530-0277.2008.00873.x, pp. 591–600. DOI: 10.1111/j.1530-0277.2008.00873.x.

[18] Luke C. Carey et al. “Ethanol decreases zinc transfer to the fetus in normal but not metallothionein-null mice”. en. In: *Alcoholism: Clinical and Experimental Research* 24.8 (Aug. 2000), pp. 1236–1240. DOI: 10.1111/j.1530-0277.2000.tb02089.x.

[19] L. C. Carey et al. “Maternal ethanol exposure is associated with decreased plasma zinc and increased fetal abnormalities in normal but not metallothionein-null mice”. en. In: *Alcoholism: Clinical and Experimental Research* 24.2 (Feb. 2000), pp. 213–219.

[20] J.C. Bremert, I.E. Dreosti, and R.S. Tulsi. “A teratogenic interaction between dietary deficiencies of zinc and folic acid in rats: An electron microscope study”. en. In: *Nutrition Research* 9.1 (Jan. 1989), pp. 105–112. DOI: 10.1016/S0271-5317(89)80108-3.

[21] Shunichi Shibazaki et al. “Copper deficiency caused by excessive alcohol consumption”. en. In: *BMJ Case Reports* (Sept. 2017), bcr–2017–220921. DOI: 10.1136/bcr-2017-220921.

[22] Hongwei Lin et al. “Dietary Copper Plays an Important Role in Maintaining Intestinal Barrier Integrity During Alcohol-Induced Liver Disease Through Regulation of the Intestinal HIF-1 Signaling Pathway and Oxidative Stress”. In: *Frontiers in Physiology* 11 (May 2020), p. 369. DOI: 10.3389/fphys.2020.00369.

[23] J H Baek and F L Cerklewski. “Influence of maternal ethanol ingestion on copper utilization during gestation and lactation in the rat”. In: *Fed. Proc., Fed. Am. Soc. Exp. Biol.;* (Mar. 1986).

[24] Cl Keen et al. “Effect of copper deficiency on prenatal development and pregnancy outcome”. en. In: *The American Journal of Clinical Nutrition* 67.5 (May 1998), 1003S–1011S. DOI: 10.1093/ajcn/67.5.1003S.

[25] Jong Ho Lee and F. L. Cerklewski. “Influence of Alcohol and Low Dietary Copper on Copper Utilization of Maternal and Offspring Liver”. en. In: *Korean Journal of Nutrition* 23.6 (1990), pp. 443–450.

[26] Kris V. Kowdley. “Alcohol intake and iron overload: Another role for hepcidin?” en. In: *Hepatology* 45.2 (Feb. 2007), pp. 541–543. DOI: 10.1002/hep.21544.

[27] Echoleah S. Rufer et al. “Adequacy of maternal iron status protects against behavioral, neuroanatomical, and growth deficits in fetal alcohol spectrum disorders”. en. In: *PLoS ONE* 7.10 (Oct. 2012). Ed. by Jonathan I. Matsui, e47499. DOI: 10.1371/journal.pone.0047499.

[28] R. Colin Carter et al. “Fetal Alcohol Exposure, Iron-Deficiency Anemia, and Infant Growth”. en. In: *Pediatrics* 120.3 (Sept. 2007), pp. 559–567. DOI: 10.1542/peds.2007-0151.

[29] Kaylee K. Helfrich et al. “Gestational Iron Supplementation Improves Fetal Outcomes in a Rat Model of Prenatal Alcohol Exposure”. en. In: *Nutrients* 14.8 (Apr. 2022), p. 1653. DOI: 10.3390/nu14081653.

[30] Ginevra Micangeli et al. “The Impact of Oxidative Stress on Pediatrics Syndromes”. en. In: *Antioxidants* 11.10 (Oct. 2022), p. 1983. DOI: 10.3390/antiox11101983.

[31] Jack Uetrecht, Denis M. Grant, and Peter G. Wells. "Biochemical mechanisms of drug toxicity". en. In: *Atkinson's Principles of Clinical Pharmacology*. Elsevier, 2022, pp. 267–302. DOI: 10.1016/B978-0-12-819869-8.00015-X.

[32] Janet Y. Uriu-Adams et al. "Influence of copper on early development: prenatal and postnatal considerations". en. In: *BioFactors* 36.2 (Mar. 2010), pp. 136–152. DOI: 10.1002/biof.85.

[33] Duygu Dee Harrison-Findik. "Role of alcohol in the regulation of iron metabolism". en. In: *World Journal of Gastroenterology* 13.37 (2007), p. 4925. DOI: 10.3748/wjg.v13.i37.4925.

[34] Patricia S. Brocardo, Joana Gil-Mohapel, and Brian R. Christie. "The role of oxidative stress in fetal alcohol spectrum disorders". en. In: *Brain Research Reviews* 67.1-2 (June 2011), pp. 209–225. DOI: 10/bqh4w4.

[35] James A. Marrs et al. "Zebrafish fetal alcohol syndrome model: effects of ethanol are rescued by retinoic acid supplement". en. In: *Alcohol* 44.7-8 (Nov. 2010), pp. 707–715. DOI: 10.1016/j.alcohol.2009.03.004.

[36] Evelyne Muggli et al. "A data driven approach to identify trajectories of prenatal alcohol consumption in an australian population-based cohort of pregnant women". en. In: *Scientific Reports* 12.1 (Dec. 2022), p. 4353. DOI: 10.1038/s41598-022-08190-4.

[37] Gretchen Bandoli et al. "Patterns of prenatal alcohol use that predict infant growth and development". en. In: *Pediatrics* 143.2 (Feb. 2019). <sub>PMCID: PMC6361345, e20182399. DOI: 10.1542/peds.2018-2399.

[38] Gretchen Bandoli et al. "Patterns of Prenatal Alcohol Exposure and Alcohol-Related Dysmorphic Features". en. In: *Alcoholism: Clinical and Experimental Research* 44.10 (Oct. 2020), pp. 2045–2052. DOI: 10.1111/acer.14430.

[39] Andrew D. Arenson et al. "Implementation of a shared data repository and common data dictionary for fetal alcohol spectrum disorders research". In: *Alcohol*. Special Issue on Fetal Alcohol Spectrum Disorders: Diagnosis and Intervention 44.7 (Nov. 2010), pp. 643–647. DOI: 10.1016/j.alcohol.2009.08.007.

[40] Sarah N. Mattson et al. "Collaborative initiative on fetal alcohol spectrum disorders: methodology of clinical projects". en. In: *Alcohol* 44.7-8 (Nov. 2010), pp. 635–641. DOI: 10.1016/j.alcohol.2009.08.005.

[41] M Russell et al. "Detecting risk drinking during pregnancy: a comparison of four screening questionnaires." en. In: *American Journal of Public Health* 86.10 (Oct. 1996), pp. 1435–1439. DOI: 10.2105/AJPH.86.10.1435.

[42] Christina D. Chambers et al. "Alcohol consumption patterns among pregnant women in the Moscow region of the russian federation". en. In: *Alcohol (Fayetteville, N.Y.)* 38.3 (Apr. 2006), pp. 133–137. DOI: 10.1016/j.alcohol.2006.06.002.

[43] Charles R. Carlson et al. "Vitamin D Deficiency in Pregnant Ukrainian Women: Effects of Alcohol Consumption on Vitamin D Status". en. In: *Journal of the American College of Nutrition* 36.1 (Jan. 2017), pp. 44–56. DOI: 10.1080/07315724.2016.1174091.

[44] K.D. Sowell et al. "Implications of altered maternal cytokine concentrations on infant outcomes in children with prenatal alcohol exposure". en. In: *Alcohol* 68 (May 2018), pp. 49–58. DOI: 10.1016/j.alcohol.2017.08.006.

[45] L.C. Sobell and Mark Sobell. "Timeline follow-back: A technique for assessing self-reported alcohol consumption". In: *Measuring Alcohol Consumption: Psychosocial and Biochemical Methods* (Jan. 1992), pp. 41–72.

[46] Joe M. McCord and Irwin Fridovich. "Superoxide Dismutase". en. In: *Journal of Biological Chemistry* 244.22 (Nov. 1969), pp. 6049–6055. DOI: 10.1016/S0021-9258(18)63504-5.

[47] R. A. Lawrence and R. F. Burk. "Glutathione peroxidase activity in selenium-deficient rat liver". en. In: *Biochemical and Biophysical Research Communications* 71.4 (Aug. 1976), pp. 952–958. DOI: 10.1016/0006-291x(76)90747-6.

[48] N. Agergaard and P. Thode Jensen. "Procedure for blood glutathione peroxidase determination in cattle and swine". en. In: *Acta Veterinaria Scandinavica* 23.4 (Dec. 1982). Number: 4 Publisher: BioMed Central, pp. 515–527. DOI: 10.1186/BF03546770.

[49] M. B. Cohen and D. L. Duvel. "Characterization of the inhibition of glutathione reductase and the recovery of enzyme activity in exponentially growing murine leukemia (L1210) cells treated with 1,3-bis(2-chloroethyl)-1-nitrosourea". en. In: *Biochemical Pharmacology* 37.17 (Sept. 1988), pp. 3317–3320. DOI: 10.1016/0006-2952(88)90645-4.

[50] K.M. Rogers and R.C. Augusteyn. "Glutathione reductase in normal and cataractous human lenses". en. In: *Experimental Eye Research* 27.6 (Dec. 1978), pp. 719–721. DOI: 10.1016/0014-4835(78)90041-6.

[51] D. R. Janero. "Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury". en. In: *Free Radical Biology & Medicine* 9.6 (1990), pp. 515–540. DOI: 10.1016/0891-5849(90)90131-2.

[52] Kunio Yagi. "Assay for blood plasma or serum". en. In: *Methods in Enzymology*. Vol. 105. Oxygen Radicals in Biological Systems. Academic Press, Jan. 1984, pp. 328–331. DOI: 10.1016/S0076-6879(84)05042-4.

[53] Cesar G. Fraga, Brian E. Leibovitz, and Al L. Tappel. "Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes". en. In: *Free Radical Biology & Medicine* 4.3 (Jan. 1988), pp. 155–161. DOI: 10.1016/0891-5849(88)90023-8.

[54] Eduardo Lissi et al. "Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements". en. In: *Free Radical Biology & Medicine* 18.2 (Feb. 1995), pp. 153–158. DOI: 10.1016/0891-5849(94)00117-3.

[55] Janice F. Wang et al. "A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage". en. In: *Journal of Nutrition* 130.8 (Aug. 2000), 2115S–2119S. DOI: 10.1093/jn/130.8.2115S.

[56] Karl H Schosinsky, H Peter Lehmann, and Myrton F Beeler. "Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride". en. In: *Clinical Chemistry* 20.12 (Dec. 1974), pp. 1556–1563. DOI: 10.1093/clinchem/20.12.1556.

[57] N Bayley. *Bayley scales of infant development (BSID-2)*. San Antonio, TX: The Psychological Corporation, 1993.

[58] Daniel J. Stekhoven and Peter Bühlmann. "MissForest—non-parametric missing value imputation for mixed-type data". In: *Bioinformatics* 28.1 (Jan. 2012), pp. 112–118. DOI: 10.1093/bioinformatics/btr597.

[59] R Core Team. *R: a language and environment for statistical computing*. en. manual. Vienna, Austria: R Foundation for Statistical Computing, 2021.

[60] Steven P. Millard. *EnvStats: an R package for environmental statistics*. en. 2nd ed. New York, NY: Springer New York, 2013.

[61] Linda K. Muthén and Bengt O. Muthén. *Mplus User's Guide*. en. Apr. 2017.

[62] Mollie E. Brooks et al. "glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling". en. In: *R Journal* 9.2 (2017), p. 378. DOI: 10.32614/RJ-2017-066.

[63] Yoav Benjamini and Yosef Hochberg. "Controlling the false discovery rate: a practical and powerful approach to multiple testing". en. In: *Journal of the Royal Statistical Society: Series B (methodological)* 57.1 (Jan. 1995), pp. 289–300. DOI: 10.1111/j.2517-6161.1995.tb02031.x.

[64] Shinichi Nakagawa. "A farewell to bonferroni: the problems of low statistical power and publication bias". en. In: *Behavioral Ecology* 15.6 (Nov. 2004), pp. 1044–1045. DOI: 10.1093/beheco/arh107.

[65] Lawrence J. Berliner and Narasimham L. Parinandi, eds. *Measuring Oxidants and Oxidative Stress in Biological Systems*. en. 1st ed. Biological Magnetic Resonance. Springer Cham, 2020.

[66] Ümit Mutlu-Türkoğlu et al. "Increased lipid and protein oxidation and DNA damage in patients with chronic alcoholism". en. In: *Journal of Laboratory and Clinical Medicine* 136.4 (Oct. 2000), pp. 287–291. DOI: 10.1067/mlc.2000.109097.

[67] Daniela Metro et al. "Effects of alcohol consumption on oxidative stress in a sample of patients recruited in a dietary center in a southern university hospital: a retrospective study". en. In: *Medicina* 58.11 (Nov. 2022), p. 1670. DOI: 10.3390/medicina58111670.

[68] P. Clot et al. "Monitoring oxidative damage in patients with liver cirrhosis and different daily alcohol intake." en. In: *Gut* 35.11 (Nov. 1994). Publisher: BMJ Publishing Group Section: Research Article, pp. 1637–1643. DOI: 10.1136/gut.35.11.1637.

[69] Ismail Laher, ed. *Systems Biology of Free Radicals and Antioxidants*. 1st ed. Berlin, Heidelberg: Springer, 2014. DOI: 10.1007/978-3-642-30018-9.

[70] Mi Yang et al. "The status of oxidative stress in patients with alcohol dependence: a meta-analysis". en. In: *Antioxidants* 11.10 (Sept. 2022), p. 1919. DOI: 10.3390/antiox11101919.

[71] Maria do Carmo de Carvalho e Martins et al. "Biological Indicators of Oxidative Stress [Malondialdehyde, Catalase, Glutathione Peroxidase, and Superoxide Dismutase] and Their Application in Nutrition". en. In: *Biomarkers in Nutrition*. Ed. by Vinood B. Patel and Victor R. Preedy. Biomarkers in Disease: Methods, Discoveries and Applications. Cham: Springer International Publishing, 2022, pp. 1–25. DOI: 10.1007/978-3-030-81304-8_49-1.

[72] Eser Ciftci, Vedat Turkoglu, and Zehra Bas. "Inhibition effect of thymoquinone and lycopene compounds on glutathione reductase enzyme activity purified from human erythrocytes". en. In: *Journal of Biomolecular Structure and Dynamics* 40.20 (Nov. 2022), pp. 10086–10093. DOI: 10.1080/07391102.2021.1939787.

[73] Paramahamsa Maturu et al. "Ethanol induced adaptive changes in blood for the pathological and toxicological effects of chronic ethanol consumption in humans". en. In: *Experimental and Toxicologic Pathology* 64.7 (Nov. 2012), pp. 697–703. DOI: 10.1016/j.etp.2011.01.002.

[74] Subir Kumar Das et al. "Oxidative stress is the primary event: effects of ethanol consumption in brain". en. In: *Indian journal of clinical biochemistry: IJCB* 22.1 (Mar. 2007), pp. 99–104. DOI: 10.1007/BF02912890.

[75] Gülberk Uçar, Başaran Demir, and Berna Uluğ. "Lipid peroxidation and antioxidant enzyme activities in erythrocytes of type I and II alcoholics". en. In: *Cell Biochemistry and Function* 23.1 (2005), pp. 29–37. DOI: 10.1002/cbf.1125.

[76] Robert M. Johnson et al. "Red cells from glutathione peroxidase-1–deficient mice have nearly normal defenses against exogenous peroxides". en. In: *Blood* 96.5 (Sept. 2000), pp. 1985–1988. DOI: 10.1182/blood.V96.5.1985.h8001985_1985_1988.

[77] Neelesh Deshpande et al. "Effect of alcohol consumption on oxidative stress markers and its role in the pathogenesis and progression of liver cirrhosis". en. In: *American Journal of Medical and Biological Research* 1.4 (Oct. 2013), pp. 99–102. DOI: 10.12691/ajmbr-1-4-3.

[78] Marge Kartau et al. "Plasma antioxidant potential measured by total radical trapping antioxidant parameter in a cohort of multiple sclerosis patients". en. In: *Brain and Behavior* 14.1 (Jan. 2024), e3377. DOI: 10.1002/brb3.3377.

[79] D.D.M. Wayner et al. "The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma". en. In: *Biochimica et Biophysica Acta (BBA) - General Subjects* 924.3 (June 1987), pp. 408–419. DOI: 10.1016/0304-4165(87)90155-3.

[80] Qiang Ma. "Role of Nrf2 in Oxidative Stress and Toxicity". en. In: *Annual Review of Pharmacology and Toxicology* 53.1 (Jan. 2013), pp. 401–426. DOI: 10.1146/annurev-pharmtox-011112-140320.

[81] Lei Yang et al. "[dynamic changes of ROS and Nrf2-related factors during erythroid development]". en. In: *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 31.1 (Feb. 2023), pp. 247–253. DOI: 10.19746/j.cnki.issn.1009-2137.2023.01.039.

[82] Kenneth T. Bogen. "Low-dose dose–response for In vitro Nrf2-ARE activation in human HepG2 cells". en. In: *Dose-Response* 15.2 (June 2017), p. 155932581769969. DOI: 10.1177/1559325817699696.

[83] Luc J. M. Bischoff et al. "A systematic analysis of Nrf2 pathway activation dynamics during repeated xenobiotic exposure". en. In: *Archives of Toxicology* 93.2 (Feb. 2019), pp. 435–451. DOI: 10.1007/s00204-018-2353-2.

[84] Ahmet Kahraman, Hamdullah Çakar, and Tülay Köken. "The protective effect of quercetin on long-term alcohol consumption-induced oxidative stress". en. In: *Molecular Biology Reports* 39.3 (Mar. 2012), pp. 2789–2794. DOI: 10.1007/s11033-011-1037-2.

[85] R. Senthilkumar, M. Sengottuvelan, and N. Nalini. "Protective effect of glycine supplementation on the levels of lipid peroxidation and antioxidant enzymes in the erythrocyte of rats with alcohol-induced liver injury". en. In: *Cell Biochemistry and Function* 22.2 (Mar. 2004), pp. 123–128. DOI: 10.1002/cbf.1062.

[86] J. Fehér et al. "Effect of free radical scavengers on superoxide dismutase (SOD) enzyme in patients with alcoholic cirrhosis". en. In: *Acta Medica Hungarica* 45.3-4 (1988), pp. 265–276.

[87] Elisabet R. Hillesund et al. "Alcohol consumption among students and its relationship with nutritional intake: a cross-sectional study". en. In: *Public Health Nutrition* 24.10 (July 2021), pp. 2877–2888. DOI: 10.1017/S1368980020004450.

[88] Janet E. Clements and Barbara B. Anderson. "Glutathione reductase activity and pyridoxine (pyridoxamine) phosphate oxidase activity in the red cell". In: *Biochimica et Biophysica Acta (BBA) - General Subjects* 632.2 (Oct. 1980), pp. 159–163. DOI: 10.1016/0304-4165(80)90073-2.

[89] J. M. Cooperman et al. "Erythrocyte glutathione reductase as a measure of riboflavin nutritional status of pregnant women and newborns". en. In: *Experimental Biology and Medicine* 143.2 (June 1973), pp. 326–328. DOI: 10.3181/00379727-143-37313.

[90] Mahtab S. Bamji. "Glutathione reductase activity in red blood cells and riboflavin nutritional status in humans". en. In: *Clinica Chimica Acta* 26.2 (Nov. 1969), pp. 263–269. DOI: 10.1016/0009-8981(69)90376-3.

[91] Signe Mosegaard et al. "Riboflavin deficiency—implications for general human health and inborn errors of metabolism". en. In: *International journal of molecular sciences* 21.11 (May 2020), p. 3847. DOI: 10.3390/ijms21113847.

[92] Sumathi Swaminathan, Tinku Thomas, and Anura V. Kurpad. "B-vitamin interventions for women and children in low-income populations:" en. In: *Current Opinion in Clinical Nutrition & Metabolic Care* 18.3 (May 2015), pp. 295–306. DOI: 10.1097/MCO.0000000000000166.

[93] Xia Jiang et al. "[effects of organic selenium supplement on glutathione peroxidase activities: a meta-analysis of randomized controlled trials]". en. In: *Wei Sheng Yan Jiu = Journal of Hygiene Research* 41.1 (Jan. 2012), pp. 120–123.

[94] Cd Thomson, Lk Ong, and Mf Robinson. "Effects of supplementation with high-selenium wheat bread on selenium, glutathione peroxidase and related enzymes in blood components of New Zealand residents". en. In: *American Journal of Clinical Nutrition* 41.5 (May 1985), pp. 1015–1022. DOI: 10.1093/ajcn/41.5.1015.

[95] E. C Schlorff, K Husain, and S. M Somani. "Dose- and time-dependent effects of ethanol on plasma antioxidant system in rat". en. In: *Alcohol* 17.2 (Feb. 1999), pp. 97–105. DOI: 10.1016/S0741-8329(98)00039-1.

[96] Carl L Keen et al. "Toxicant exposure and trace element metabolism in pregnancy". en. In: *Environmental Toxicology and Pharmacology* 4.3 (Dec. 1997), pp. 301–308. DOI: 10.1016/S1382-6689(97)10028-X.

[97] L. C. Carey et al. "Ethanol decreases zinc transfer to the fetus in normal but not metallothionein-null mice". en. In: *Alcoholism: Clinical and Experimental Research* 24.8 (Aug. 2000), pp. 1236–1240.

[98] Christopher Duggan et al. "Plasma Zinc Concentrations Are Depressed during the Acute Phase Response in Children with Falciparum Malaria". en. In: *The Journal of Nutrition* 135.4 (Apr. 2005), pp. 802–807. DOI: 10.1093/jn/135.4.802.

[99] George P. Daston et al. "Altered Zn status by -hederin in the pregnant rat and its relationship to adverse developmental outcome". en. In: *Reproductive Toxicology* 8.1 (Jan. 1994), pp. 15–24. DOI: 10.1016/0890-6238(94)90063-9.

[100] Allison L Fisher and Elizabeta Nemeth. "Iron homeostasis during pregnancy". en. In: *The American Journal of Clinical Nutrition* 106 (Dec. 2017), 1567S–1574S. DOI: 10.3945/ajcn.117.155812.

[101] A. Shenkin. "Trace elements and inflammatory response: implications for nutritional support". en. In: *Nutrition (Burbank, Los Angeles County, Calif.)* 11.1 Suppl (1995), pp. 100–105.

[102] Bakytzhan Bakhautdin et al. "Protective role of macrophage-derived ceruloplasmin in inflammatory bowel disease". en. In: *Gut* 62.2 (Feb. 2013), pp. 209–219. DOI: 10.1136/gutjnl-2011-300694.

[103] M.B. Pepys. "The acute phase response and C-reactive protein". en. In: *Oxford Textbook of Medicine*. Ed. by David A. Warrell, Timothy M. Cox, and John D. Firth. Oxford University Press, May 2010, pp. 1752–1759. DOI: 10.1093/med/9780199204854.003.121201.

[104] Wolfgang Koenig. "High-sensitivity C-reactive protein and atherosclerotic disease: from improved risk prediction to risk-guided therapy". en. In: *International Journal of Cardiology* 168.6 (Oct. 2013), pp. 5126–5134. DOI: 10.1016/j.ijcard.2013.07.113.

[105] Ana M. Adamo and Patricia I. Oteiza. "Zinc deficiency and neurodevelopment: the case of neurons". en. In: *BioFactors* 36.2 (Mar. 2010), pp. 117–124. DOI: 10.1002/biof.91.

[106] Charles S Lieber. "ALCOHOL: Its Metabolism and Interaction With Nutrients". en. In: *Annu Rev Nutr* (2000), 20:395–430. DOI: 10.1146/annurev.nutr.20.1.395.

[107] L. Glória et al. "Nutritional deficiencies in chronic alcoholics: relation to dietary intake and alcohol consumption". eng. In: *The American Journal of Gastroenterology* 92.3 (Mar. 1997), pp. 485–489.

[108] Luke C. Carey et al. "Zinc Supplementation at the Time of Ethanol Exposure Ameliorates Teratogenicity in Mice". en. In: *Alcoholism: Clinical and Experimental Research* 27.1 (Jan. 2003), pp. 107–110. DOI: 10.1111/j.1530-0277.2003.tb02729.x.

[109] Juna V. Konomi et al. "Zinc insufficiency mediates ethanol-induced alveolar macrophage dysfunction in the pregnant female mouse". en. In: *Alcohol and Alcoholism* 50.1 (Jan. 2015), pp. 30–38. DOI: 10.1093/alcalc/agu073.

[110] Zhanxiang Zhou et al. "Zinc supplementation inhibits hepatic apoptosis in mice subjected to a long-term ethanol exposure". en. In: *Experimental Biology and Medicine* 233.5 (May 2008), pp. 540–548. DOI: 10.3181/0710-RM-265.

[111] George N. Ioannou et al. "The effect of alcohol consumption on the prevalence of iron overload, iron deficiency, and iron deficiency anemia". en. In: *Gastroenterology* 126.5 (May 2004), pp. 1293–1301. DOI: 10.1053/j.gastro.2004.01.020.

[112] Domingo J. Piñero et al. "Variations in Dietary Iron Alter Brain Iron Metabolism in Developing Rats". In: *The Journal of Nutrition* 130.2 (Feb. 2000), pp. 254–263. DOI: 10.1093/jn/130.2.254.

[113] M. Roncagliolo et al. "Evidence of altered central nervous system development in infants with iron deficiency anemia at 6 mo: delayed maturation of auditory brainstem responses". en. In: *The American Journal of Clinical Nutrition* 68.3 (Sept. 1998), pp. 683–690. DOI: 10.1093/ajcn/68.3.683.

[114] John Beard, Keith M. Erikson, and Byron C. Jones. "Neonatal Iron Deficiency Results in Irreversible Changes in Dopamine Function in Rats". In: *The Journal of Nutrition* 133.4 (Apr. 2003), pp. 1174–1179. DOI: 10.1093/jn/133.4.1174.

[115] Kaylee Helfrich et al. "The clinical iron supplement fer-In-sol improves fetal iron status and brain weight in rats prenatally exposed to alcohol". en. In: *Current Developments in Nutrition* 4 (June 2020), nzaa050_010. DOI: 10.1093/cdn/nzaa050_010.

[116] R. Moirand et al. "Increase in glycosylated and nonglycosylated serum ferritin in chronic alcoholism and their evolution during alcohol withdrawal". en. In: *Alcoholism: Clinical and Experimental Research* 15.6 (Nov. 1991), pp. 963–969. DOI: 10.1111/j.1530-0277.1991.tb05196.x.

[117] M. Välimäki, M. Härkönen, and R. Ylikahri. "Serum ferritin and iron levels in chronic male alcoholics before and after ethanol withdrawal". en. In: *Alcohol and Alcoholism* (Aug. 1983). DOI: 10.1093/oxfordjournals.alcalc.a044371.

[118] R. Mokand et al. "Regulation of ferritin expression by alcohol in a human hepatoblastoma cell line and in rat hepatocyte cultures:" en. In: *European Journal of Gastroenterology and Hepatology* 8.1 (Jan. 1996), p. 95. DOI: 10.1097/00042737-199601000-00021.

[119] Nikhil Kumar Kotla et al. "The role of ferritin in health and disease: recent advances and understandings". en. In: *Metabolites* 12.7 (July 2022). Number: 7 Publisher: Multidisciplinary Digital Publishing Institute, p. 609. DOI: 10.3390/metabo12070609.

[120] Duygu Dee Harrison-Findik et al. "Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression*". en. In: *Journal of Biological Chemistry* 281.32 (Aug. 2006), pp. 22974–22982. DOI: 10.1074/jbc.M602098200.

[121] R. Nordmann. "Alcohol and antioxidant systems". en. In: *Alcohol and Alcoholism (Oxford, Oxfordshire)* 29.5 (Sept. 1994), pp. 513–522.

[122] H. Eugene Hoyme et al. "Updated clinical guidelines for diagnosing fetal alcohol spectrum disorders". en. In: *Pediatrics* 138.2 (Aug. 2016), e20154256. DOI: 10.1542/peds.2015-4256.

[123] R Colin Carter et al. "Gestational weight gain and dietary energy, iron, and choline intake predict severity of fetal alcohol growth restriction in a prospective birth cohort". en. In: *The American Journal of Clinical Nutrition* 116.2 (Aug. 2022), pp. 460–469. DOI: 10.1093/ajcn/nqac101.

[124] Julie M. Hasken et al. "Maternal dietary intake among alcohol-exposed pregnancies is linked to early infant physical outcomes in South Africa". en. In: *Reproductive Toxicology* 121 (Oct. 2023), p. 108467. DOI: 10.1016/j.reprotox.2023.108467.

[125] Claire D. Coles et al. "Dose and Timing of Prenatal Alcohol Exposure and Maternal Nutritional Supplements: Developmental Effects on 6-Month-Old Infants". en. In: *Maternal and Child Health Journal* 19.12 (Dec. 2015), pp. 2605–2614. DOI: 10.1007/s10995-015-1779-x.

[126] J.A. Kable et al. "The impact of micronutrient supplementation in alcohol-exposed pregnancies on information processing skills in Ukrainian infants". en. In: *Alcohol* 49.7 (Nov. 2015), pp. 647–656. DOI: 10.1016/j.alcohol.2015.08.005.

[127] J.A. Kable et al. "The impact of micronutrient supplementation in alcohol-exposed pregnancies on reaction time responses of preschoolers in Ukraine". en. In: *Alcohol* 99 (Mar. 2022), pp. 49–58. DOI: 10.1016/j.alcohol.2021.12.002.

[128] Claire D. Coles et al. "Measurement of neurodevelopmental effects of prenatal alcohol exposure in Ukrainian preschool children". en. In: *Child Neuropsychology* 27.8 (Nov. 2021), pp. 1088–1103. DOI: 10.1080/09297049.2021.1919298.

Chapter 4

Maternal Acute Phase Response to Prenatal Alcohol Exposure: Associations with Nutrient Dysregulation, Infant Sex, and Developmental Outcomes.

4.1 Introduction

4.1.1 Background on FASD and PAE

PAE is a significant preventable cause of neurodevelopmental disabilities, known as FASD[1]. Lemoine first characterized these conditions in 1968[2]. Jones and Smith, in 1973, coined the term FAS[3]. Global prevalence estimates indicate FASD affects approximately 7.7 per 1,000 individuals[4]. In the United States, estimated prevalence among school-aged children ranges from 1.4% to 8.4%[5–8]. Higher rates occur in specific high-risk populations[9]. Alcohol acts as a teratogen, crossing the placenta to disrupt fetal development, particularly impacting

the vulnerable CNS[10–14]. FASD manifestation and severity relate to the dose, pattern, and timing of alcohol exposure. Genetic predispositions and maternal physiological factors can modulate these outcomes[15–18]. Maternal nutritional status and oxidative stress levels further modify fetal susceptibility to alcohol-induced damage[19–21]. Understanding these complex interactions is critical for mitigating FASD risk.

4.1.2 Role of Maternal Nutrition and Oxidative Stress

Suboptimal maternal nutrition can worsen alcohol's teratogenic effects, increasing FASD risk beyond the independent contributions of PAE or nutrient deficiency alone[19]. Oxidative stress, an imbalance favoring ROS production over antioxidant defenses, appears central to PAE-induced fetal damage[20,21]. Alcohol metabolism generates ROS, potentially overwhelming endogenous antioxidants and damaging developing tissues, particularly the fetal brain[22];[21]. Deficiencies in nutrients, such as zinc or copper required for antioxidant enzymes like CuZnSOD, compromise these defenses[19]. PAE can induce secondary nutritional deficits. The alcohol-triggered APR involves inflammation-mediated hepatic zinc sequestration. This process reduces zinc availability for the fetus[19,23,24]. Animal studies demonstrated that marginal zinc status exacerbated alcohol's teratogenic effects, while zinc supplementation offered protection[25,26],

4.1.3 Challenges in PAE Research and Alcohol Trajectory Analysis

Investigating these nutrition and PAE interactions in humans faces difficulties related to the diverse patterns of alcohol consumption during pregnancy. Binary classifications like drinker versus non-drinker obscure critical details of quantity, frequency, and timing. Previous research indicated that analyzing longitudinal alcohol consumption trajectories predicted differential risks for adverse child outcomes more effectively than static categories[27,28]. Yet, a

gap remains in understanding how these dynamic patterns of PAE interact with the mother's changing nutritional status throughout gestation to influence fetal development.

4.1.4 Study Rationale and Objectives

Our prior work integrated detailed alcohol trajectory modeling with maternal biomarker measurements (Chapter 3). Those analyses demonstrated that distinct maternal biochemical profiles, reflecting oxidative stress, antioxidant capacity, nutrient status, and inflammation, were associated with different gestational alcohol intake trajectories. Women following patterns of higher sustained drinking showed greater physiological and nutritional disruption. This suggests that the specific pattern of PAE, not just the presence or absence of drinking, interacts with the maternal biochemical environment. This study extends that work by examining associations between distinct maternal alcohol consumption trajectories, concurrent maternal biomarkers of nutritional and oxidative stress status during pregnancy, and resulting infant anthropometric and neurodevelopmental outcomes. The objective is to determine how the interplay between specific PAE patterns and maternal physiological state relates to infant outcomes.

4.2 Materials and Methods

4.2.1 Study Design and Participant Recruitment

Data originated from a prospective cohort study within the CIFASD, funded by the National Institute on Alcohol Abuse and Alcoholism, conducted from April 2008 to August 2012 at two prenatal care facilities in Western Ukraine: the Rivne Regional Medical Diagnostic Center and the Khmelnytsky Perinatal Center. These sites participated in OMNI-Net, a network for birth defects prevention. The CIFASD parent study aimed to characterize FASD, develop improved diagnostic and prevention strategies, and investigate maternal nutrition as a modifying factor; detailed methods and scope of CIFASD studies appear elsewhere[29,30].

Institutional review boards at the University of California San Diego and Lviv Medical University in Ukraine approved the study protocol. All participants provided written informed consent before enrollment. Pregnant women attending routine prenatal visits at these centers were screened for alcohol consumption. Women entered an AE group if they reported alcohol consumption during the month near conception or the most recent pregnancy month meeting one of the following criteria: weekly binge-drinking episodes (four to five or more standard alcoholic drinks per occasion), five or more episodes of three to four standard drinks, or ten or more episodes of one to two standard drinks. Upon recruitment of an AE participant, the subsequent woman meeting criteria for low or no alcohol exposure (consuming fewer than two drinks per occasion, two or fewer drinks per week near conception, and no alcohol consumption during the most recent pregnancy month) entered a comparison group.

Sociodemographic, Lifestyle, and Clinical Data Collection

Data acquisition consisted of maternal interviews and biological sampling at enrollment and a follow-up visit near 32 weeks gestation. Interviews recorded demographics, BMI prior to pregnancy, SES (Hollingshead ratings), health behaviors like tobacco use, existing supplement use, and pregnancy characteristics. Maternal blood samples were obtained at enrollment and the third-trimester visit for biomarker analysis.

Gestational Visit Scheduling and Data Handling

We categorized gestational timing for maternal interviews using the method of Sowell et al. (2018); this method separated visits into early/mid pregnancy (< 26 weeks) or late pregnancy (≥ 26 weeks)[31]. If a participant attended two visits within the early/mid pregnancy period, we excluded the second visit to prevent data duplication.

Assessment of Prenatal Alcohol Exposure

Alcohol Consumption Quantification We quantified maternal alcohol consumption using a TLFB method[32] for women reporting any lifetime drinking. The TLFB captured day-by-day alcohol intake (type, quantity, frequency) for a typical week near conception, for the two weeks preceding enrollment, and changes reported at a follow-up visit (approximately 32 weeks' gestation), with quantities converted to absolute ounces of alcohol (ozAA) per day (one standard drink equaled 0.5 ozAA).

Latent Class Trajectory Modeling To construct individual consumption trajectories, we assigned the reported consumption at conception from week 0 until the week preceding pregnancy recognition, applied the consumption level at pregnancy recognition from that week until the follow-up visit, and used the follow-up visit measurement from that point through delivery, creating a continuous series of weekly alcohol intake values. Details regarding inclusion criteria, such as the exclusion of twin pregnancies and participants with insufficient data for trajectory construction, are described previously (Chapter 3). For modeling, we aggregated weekly data into monthly averages to reduce data dimensionality and collinearity. We then used Mplus (version 8.11)[33] to estimate a GMM identifying latent classes of similar alcohol consumption trajectories among individuals reporting any alcohol intake; women reporting no alcohol consumption formed an a priori defined subset and were excluded from this modeling. The GMM defined classes with two growth factors (intercept and slope) across seven measurement points (conception, and months 1, 2, 3, 4, 7, and 10). Within each class, the model permitted intercept and slope to vary, capturing individual deviations. We fixed the covariance between intercept and slope at zero, meaning an individual's initial consumption level did not predict the rate of change, a restriction that aided convergence and model estimation with missing information. The Mplus syntax appears in Supplemental Code Listing A.1. A four-class solution was selected based on its balance of AIC, BIC, and its alignment with trajectories from previous studies of this population[27,28]

(Figure 4.1). The four latent class prenatal alcohol consumption trajectories and the abstained group were: Abstain (N=328), Low (N=70), ModDisc (N=99), HighMod (N=128), and VHigh (N=40).

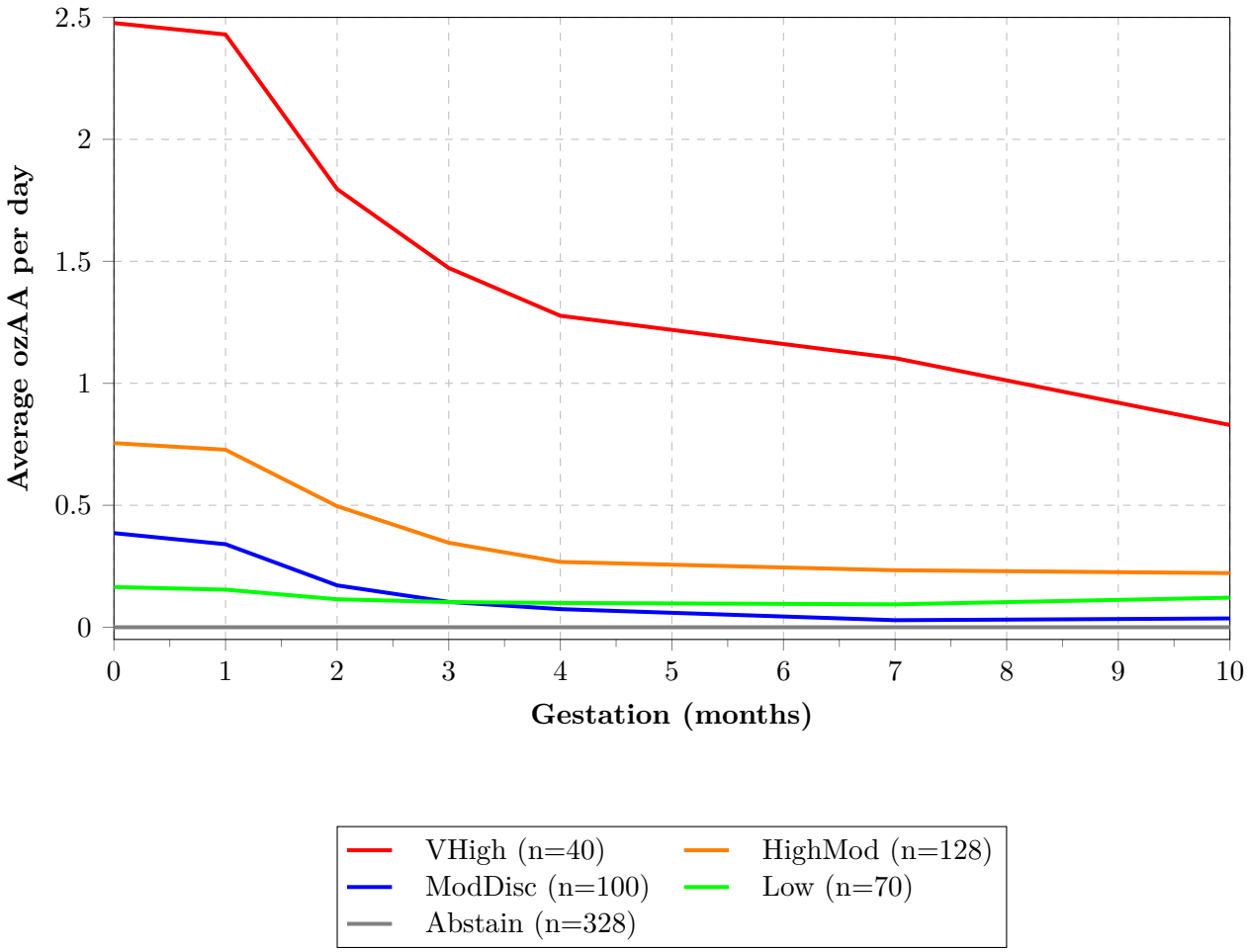

Figure 4.1: Mean prenatal alcohol consumption trajectories (ounces of absolute alcohol [ozAA]/day) for the four identified latent classes and the Abstained group.

Maternal Biomarker Assays

Specifics for these maternal biological analyses were described previously (Chapter 3). Erythrocyte SOD activity was determined spectrophotometrically by its inhibition of pyrogallol autooxidation[34]. Erythrocyte GPx activity was quantified using a coupled enzymatic reaction monitoring NADPH oxidation[35,36]. Erythrocyte GRed activity was measured via a

kinetic spectrophotometric assay observing NADPH oxidation during GSSG reduction[37,38]. Plasma TBARS were quantified fluorometrically after reaction of plasma components with TBA and subsequent extraction[39–41]. Plasma TRAP was assessed with a chemiluminescence-based assay measuring the lag phase of luminol degradation initiated by ABAP, with calibration against Trolox[42,43]. Plasma zinc, iron, and copper concentrations were determined using ICP-OES. Plasma CP oxidase activity was quantified by a kinetic spectrophotometric assay measuring o-dianisidine dihydrochloride oxidation[44]. A solid-phase chemiluminescent immunometric assay quantified plasma hsCRP concentrations, and a solid-phase, two-site chemiluminescent immunometric assay quantified plasma ferritin; both assays utilized an IMMULITE analyzer. Plasma TfR concentrations were measured with a quantitative sandwich enzyme immunoassay.

4.2.2 Infant Outcome Assessment

Infant anthropometric measures at birth (birthweight in grams, length in cm, head circumference in cm, gestational age at birth in weeks) were obtained from medical records. Neurodevelopment was evaluated at approximately 6 and 12 months postpartum. Trained psychologists, blinded to maternal alcohol exposure and intervention group status, administered the BSID-II, Second Edition[45]. This assessment provided standardized scores (population mean=100, SD=15), age-corrected for infants born before 37 weeks gestation, for the MDI and the PDI. The MDI assessed early cognitive and language abilities, including knowledge, problem-solving, and memory. The PDI evaluated motor skills, such as body control and the coordination of large and fine muscle movements.

4.2.3 Data Management and Pre-processing

Covariate Definition and Imputation

We created a composite classification for maternal smoking from self-reported smoking status and reported daily smoking counts. This classification identified individuals who reported no smoking and quantified daily smoking for those who smoked.

Missing covariate data were imputed using the `missForest` nonparametric algorithm (version 1.5)[46] in R version 4.4.2[47], a method that accommodates mixed-type data and utilizes all available measures; biomarker data and alcohol intake were not imputed. The specifics of the twelve imputed covariates, each with 1-2% missing data (including pre-pregnancy BMI), alongside their individual mean squared error estimates from the imputation procedure (500 trees, 100 iterations), are detailed in Chapter 3; the resulting imputations remained within original data ranges and were near the mean and median of non-missing observations.

Outlier Identification and Winsorization

Outliers across covariates and biomarkers were identified visually (Q–Q plots, histograms) and with Rosner's test in R using the `EnvStats` package (version 3.0.0)[48]; identified outliers were winsorized by substituting the next highest or lowest non-outlier value. This approach was applied to daily alcohol consumption data (at conception, enrollment, and a follow-up pregnancy visit) and to second-visit data for TBARS and plasma iron. Chapter 3 provides detailed descriptions of these outliers, their test statistics, and replacement values; three alcohol outliers at conception, one at enrollment, and three at follow-up, plus one outlier for TBARS and one for plasma iron, were winsorized to aid model convergence.

4.2.4 Statistical Analysis

Alcohol Trajectories, Infant Sex, and Infant Outcomes

Alcohol Trajectories, Infant Sex, and Maternal Biomarker Status

We examined associations of maternal alcohol consumption trajectories and infant sex with maternal biomarkers at two pregnancy periods (early/mid and late pregnancy), including TBARS, erythrocyte GPx, erythrocyte GRed, erythrocyte SOD, TRAP, hsCRP, CP, copper, zinc, iron, ferritin, and TfR. A series of GLMs modeled each maternal biomarker as an outcome for each pregnancy period, with models including maternal alcohol consumption trajectory, infant sex, and an interaction term between alcohol trajectory and infant sex. All models were adjusted for covariates: gestational age at the visit, pre-pregnancy BMI, interview location, marijuana use during pregnancy, maternal age at enrollment, maternal years of education, Hollingshead SES, total smoking during pregnancy, and gravidity. We fitted models using the `glmmTMB` package (version 1.1.10)[49] in R (version 4.4.2)[47]. Pairwise comparisons evaluated differences in maternal biomarker levels across alcohol trajectory groups stratified by infant sex, and differences between sexes within each alcohol trajectory group. P-values from these multiple comparisons were adjusted using the BH procedure, and statistical significance was set at an adjusted p-value < 0.05.

Maternal Physiological Status and Infant Outcomes

To investigate the relationships between maternal physiological status during pregnancy and infant outcomes, a series of GLMs was constructed. These models examined associations of maternal biomarkers, measured separately during early/mid-pregnancy and late pregnancy, with infant anthropometric measures at birth and neurodevelopmental scores (MDI and PDI from the BSID-II, Second Edition) at 6 and 12 months postpartum. For each maternal biomarker and child outcome pair, the GLM included the specific maternal biomarker, alcohol consumption trajectory, and infant sex as primary predictors. Each model incorpo-

rated a three-way interaction term (maternal biomarker * alcohol consumption trajectory * infant sex) to assess differential effects. Models adjusted for maternal age at enrollment, Hollingshead SES, and total smoking during pregnancy. We fitted models using the `glmmTMB` package (version 1.1.10)[49] in R (version 4.4.2)[47]. Post-hoc analyses of models examined the slope of the maternal biomarker's association with the outcome by: 1) comparing slopes between infant sexes within each maternal alcohol consumption trajectory group; 2) comparing slopes between maternal alcohol consumption trajectory groups within each infant sex. P-values for multiple comparisons in post-hoc analyses were adjusted using the BH procedure.

To account for multiple comparisons, we applied the Benjamini-Hochberg procedure to control the FDR[50]. This method, which adjusts p-values based on their rank, was selected over the Bonferroni correction to mitigate the increased risk of Type II errors associated with the Bonferroni approach and to avoid overcorrecting non-significant results[51]. Both uncorrected p-values and FDR-adjusted q-values are presented.

4.3 Results

4.3.1 Alcohol Trajectories, Infant Sex, and Infant Outcomes

We used GLMs to assess associations of maternal alcohol consumption trajectories and infant sex with infant developmental outcomes. Each model evaluated main effects of alcohol trajectory, infant sex, and their interaction on infant anthropometric measures at birth (birthweight, length, head circumference) and neurodevelopmental scores (MDI and PDI) at 6 and 12 months. Analyses adjusted for maternal age at enrollment, SES, total smoking during pregnancy, gestational age at birth, and maternal MVM supplementation status.

The alcohol trajectory-infant sex interaction was statistically significant ($p < 0.05$) for birthweight, infant length at birth, head circumference at birth, 6-month MDI, 6-month PDI, 12-month MDI, and 12-month PDI. Post-hoc analyses for these outcomes showed differential

effects of alcohol exposure patterns between male and female infants.

4.3.2 Alcohol Trajectories, Infant Sex, and Maternal Biomarker Status

GLMs assessed interactions between maternal alcohol consumption trajectories and infant sex on maternal biomarker levels. These models evaluated if the relationship between alcohol exposure patterns and maternal biochemical status differed for mothers carrying male versus female fetuses. Results for biomarkers that showed a significant interaction effect, alongside iron, are included below.

Maternal Erythrocyte GPx Activity

An examination of maternal erythrocyte GPx activity showed that its association with maternal alcohol consumption trajectory differed by infant sex. This sex-specific pattern in the relationship between alcohol trajectory and GPx levels appeared during early/mid pregnancy ($\beta = 0.136$; SE = 0.052; p = 0.008). The pattern continued into late pregnancy ($\beta = 0.208$; SE = 0.047; $p < 0.001$).

Sex Differences in Maternal Erythrocyte GPx Activity Maternal erythrocyte GPx activity differed between mothers carrying male versus female fetuses within certain alcohol consumption trajectory groups (Table 4.1).

During early/mid pregnancy, mothers carrying male fetuses in the VHigh alcohol consumption trajectory group had lower GPx levels compared to mothers carrying female fetuses in the same trajectory group (p = 0.026) (Table 4.1). Other trajectory groups did not show sex-specific differences in GPx levels at this time point. During late pregnancy, mothers carrying male fetuses had lower GPx levels than those carrying female fetuses within the Abstain (p = 0.034), HighMod ($p < 0.001$), and VHigh (p = 0.002) trajectory groups.

Maternal Erythrocyte GPx Activity Across Alcohol Trajectories Stratified by Infant Sex For mothers carrying male fetuses during early/mid pregnancy, those in the VHigh trajectory group showed lower GPx levels compared to those in the Abstain group ($p = 0.002$; $q = 0.007$), the Low group ($p < 0.001$; $q = 0.004$), and the ModDisc group ($p < 0.001$; $q = 0.004$) (Table 4.1). The GPx levels in the VHigh group were lower than in the HighMod group ($p = 0.024$; $q = 0.060$).

During late pregnancy, among mothers carrying male fetuses, those in the HighMod trajectory group had lower GPx levels than those in the Abstain group ($p < 0.001$; $q = 0.006$) and the Low group ($p = 0.009$; $q = 0.047$). Their GPx levels were lower compared to the ModDisc group ($p = 0.021$; $q = 0.061$).

For mothers carrying female fetuses, differences in GPx levels across alcohol consumption trajectory groups during early/mid pregnancy were not found after BH adjustment for multiple comparisons. During late pregnancy, among mothers carrying female fetuses, those in the VHigh trajectory group had higher GPx levels compared to those in the Abstain group ($p = 0.008$; $q = 0.076$) and the Low group ($p = 0.046$; $q = 0.114$). After BH adjustment, these differences were not statistically significant. Other comparisons across trajectory groups for mothers carrying female fetuses at this time point showed no differences after BH adjustment.

Table 4.1: Maternal Erythrocyte Glutathione Peroxidase (GPx) Activity by Prenatal Alcohol Exposure Trajectory, Pregnancy Timing, and Infant Sex

		Prenatal Alcohol Exposure Trajectory				
Timing	**Sex**	Abstain	Low	ModDisc	HighMod	VHigh
Early/Mid	Male	15.65 (0.03)^{a}	16.35 (0.05)^{a}	16.22 (0.04)^{a}	15.14 (0.04)^{ab}	13.27 (0.06)^{b,†}
	Female	15.53 (0.03)^{a}	15.11 (0.05)^{a}	15.21 (0.04)^{a}	15.49 (0.04)^{a}	15.53 (0.06)^{a†}
Late	Male	16.54 (0.03)^{a†}	16.52 (0.04)^{a}	16.14 (0.04)^{ab}	14.68 (0.04)^{b†}	14.76 (0.05)^{ab†}
	Female	15.76 (0.03)^{a†}	16.00 (0.05)^{a}	15.91 (0.04)^{a}	16.90 (0.03)^{a†}	17.94 (0.05)^{a†}

Values are mean (SE) U/g Hb.

^{a,b} Within each row, means not sharing a common letter differ significantly (BH-adjusted $p < 0.05$).

† Significant sex difference within trajectory group and timing ($p < 0.05$).

Maternal Plasma Copper

The analysis of maternal plasma copper levels showed differing patterns of association with maternal alcohol consumption trajectory and infant sex at distinct stages of pregnancy. During early/mid pregnancy, the interaction between maternal alcohol consumption trajectory and infant sex on maternal plasma copper levels was marginal (β = -0.139; SE = 0.077; p = 0.073). In late pregnancy, a significant interaction was noted between alcohol trajectory and infant sex (β = -0.189; SE = 0.091; p = 0.038).

Sex Differences in Maternal Plasma Copper During early/mid and late pregnancy, sex differences in maternal plasma copper levels within any of the alcohol trajectory groups were not observed after BH adjustment (Table 4.2).

Maternal Plasma Copper Across Alcohol Trajectories Stratified by Infant Sex For mothers carrying male fetuses, differences in plasma copper levels across the alcohol consumption trajectory groups during either early/mid pregnancy or late pregnancy were not observed after BH adjustment (Table 4.2).

For mothers carrying female fetuses during early/mid pregnancy, those in the VHigh trajectory group had lower copper levels compared to those in the Abstain group ($p < 0.001$; $q = 0.003$), the Low group ($p = 0.011$; $q = 0.028$), the ModDisc group ($p < 0.001$; $q = 0.003$), and the HighMod group (p = 0.003; q = 0.008) (Table 4.2). These differences remained after BH adjustment.

During late pregnancy, among mothers carrying female fetuses, those in the VHigh trajectory group exhibited lower copper levels compared to those in the Abstain group (p = 0.001; q = 0.009), the ModDisc group (p = 0.003; q = 0.009), and the HighMod group (p = 0.003; q = 0.009) (Table 4.2). Lower copper in the VHigh group compared to the Low group was also observed (p = 0.030; q = 0.074); this comparison was marginal after BH adjustment.

Table 4.2: Maternal Plasma Copper Levels by Prenatal Alcohol Exposure Trajectory, Pregnancy Timing, and Infant Sex

		Prenatal Alcohol Exposure Trajectory				
Timing	**Sex**	Abstain	Low	ModDisc	HighMod	VHigh
Early/Mid	Male	1.65 (0.05)[a]	1.61 (0.06)[a]	1.61 (0.06)[a]	1.58 (0.06)[a]	1.50 (0.09)[a]
	Female	1.68 (0.05)[a]	1.62 (0.07)[a]	1.71 (0.06)[a]	1.66 (0.06)[a]	1.36 (0.09)[b]
Late	Male	1.85 (0.06)[a]	1.86 (0.09)[a]	1.83 (0.08)[a]	1.73 (0.07)[a]	1.70 (0.11)[a]
	Female	1.88 (0.06)[a]	1.80 (0.09)[ab]	1.87 (0.08)[a]	1.87 (0.07)[a]	1.53 (0.11)[b]

Values are mean (SE) mg/L.

[a,b] Within each row, means not sharing a common letter differ significantly (BH-adjusted $p < 0.05$).

[†] Significant sex difference within trajectory group and timing ($p < 0.05$).

Maternal Plasma Zinc

Analysis of maternal zinc levels showed differing associations with maternal alcohol consumption trajectory and infant sex across pregnancy stages. During early/mid pregnancy, interaction effects between maternal alcohol consumption trajectory and infant sex on maternal zinc levels were not found (β = -0.011; SE = 0.036; p = 0.764). In late pregnancy, an interaction appeared for alcohol trajectory and infant sex (β = -0.079; SE = 0.028; p = 0.004). These observations indicated that the pattern of association between alcohol consumption trajectories and maternal zinc levels in late pregnancy differed for mothers carrying male versus female fetuses.

Sex Differences in Maternal Plasma Zinc Maternal zinc levels between mothers carrying male versus female fetuses were examined within alcohol consumption trajectory groups. During early/mid pregnancy, sex differences in maternal zinc levels within any of the alcohol trajectory groups were not observed after BH adjustment.

During late pregnancy, mothers carrying male fetuses in the VHigh alcohol consumption trajectory group had higher zinc levels compared to mothers carrying female fetuses in the same trajectory group (p = 0.013) (Table 4.3). Other trajectory groups did not show sex-specific differences in zinc levels at this time point.

Maternal Zinc Across Alcohol Trajectories Stratified by Infant Sex Maternal zinc levels across different alcohol consumption trajectories were examined separately for each infant sex during late pregnancy.

During late pregnancy, among mothers carrying female fetuses, those in the VHigh trajectory group showed lower zinc levels compared to those in the Abstain group ($p < 0.001$; $q = 0.002$), the Low group ($p = 0.001$; $q = 0.003$), the ModDisc group ($p < 0.001$; $q < 0.001$), and the HighMod group ($p < 0.001$; $q < 0.001$) (Table 4.3). These differences remained after BH adjustment. Among mothers carrying male fetuses, differences in plasma zinc levels across the alcohol consumption trajectory groups were not observed after BH adjustment.

Table 4.3: Maternal zinc Levels by Prenatal Alcohol Exposure Trajectory, Pregnancy Timing, and Infant Sex

		Prenatal Alcohol Exposure Trajectory				
Timing	**Sex**	Abstain	Low	ModDisc	HighMod	VHigh
Early/Mid	Male	0.63 (0.02)[a]	0.64 (0.02)[a]	0.64 (0.02)[a]	0.64 (0.02)[a]	0.56 (0.03)[b]
	Female	0.65 (0.02)[a]	0.64 (0.02)[a]	0.65 (0.02)[a]	0.64 (0.02)[a]	0.56 (0.04)[a]
Late	Male	0.57 (0.02)[a]	0.56 (0.02)[a]	0.57 (0.02)[a]	0.57 (0.02)[a]	0.55 (0.03)[a,†]
	Female	0.55 (0.02)[a]	0.56 (0.03)[a]	0.59 (0.02)[a]	0.59 (0.02)[a]	0.45 (0.03)[b,†]

Values are mean (SE) mg/L.
[a,b] Within each row, means not sharing a common letter differ significantly (BH-adjusted $p < 0.05$).
[†] Significant sex difference within trajectory group and timing ($p < 0.05$).

Maternal Plasma Iron

Analysis of maternal plasma iron levels showed minimal interaction effects between maternal alcohol consumption trajectory and infant sex during early/mid pregnancy or late pregnancy (Table 4.4). This indicated the relationship between maternal alcohol consumption patterns and maternal iron levels did not differ for mothers carrying male versus female fetuses.

Table 4.4: Maternal Plasma Iron Levels by Prenatal Alcohol Exposure Trajectory, Pregnancy Timing, and Infant Sex

		Prenatal Alcohol Exposure Trajectory				
Timing	**Sex**	Abstain	Low	ModDisc	HighMod	VHigh
Early/Mid	Male	0.86 (0.05)[a]	0.87 (0.08)[b]	1.04 (0.06)[ab]	0.96 (0.07)[ab]	0.94 (0.10)[ab]
	Female	0.90 (0.05)	0.99 (0.07)	0.97 (0.07)	0.99 (0.06)	1.05 (0.12)
Late	Male	0.74 (0.07)	0.77 (0.09)	0.80 (0.09)	0.79 (0.07)	0.82 (0.11)
	Female	0.72 (0.07)	0.75 (0.10)	0.75 (0.09)	0.80 (0.08)	0.64 (0.12)

Values are mean (SE) mg/L.
[a,b] Within each row, means not sharing a common letter differ significantly (BH-adjusted $p < 0.05$).

4.3.3 Interactive Effects of Maternal Biomarkers, Prenatal Alcohol Exposure Trajectories, and Infant Sex on Infant Outcomes

Erythrocyte Antioxidant Enzymes (GPx and SOD)

Late Pregnancy Erythrocyte GPx Activity and 6-Month MDI Scores Analysis of the relationship between maternal erythrocyte GPx activity and infant MDI scores at 6 months showed significant sex differences within alcohol trajectory groups and distinct patterns across drinking profiles when stratified by infant sex.

Significant sex differences emerged within specific alcohol trajectory groups. In the Low group, male infants (β = -0.625; SE = 0.419) showed a substantially more negative GPx-MDI relationship than female infants (β = 0.712; SE = 0.459; p = 0.032) (Figure 4.2). This sex divergence was also observed in the VHigh group, where male infants exhibited a negative association (β = -2.672; SE = 0.506) while female infants demonstrated a positive relationship (β = 1.546; SE = 0.584; $p < 0.001$).

When comparing trajectory groups within males, the VHigh group demonstrated a significantly more negative GPx-MDI relationship than all other drinking patterns. The GPx-MDI relationship in the VHigh group (β = -2.672; SE = 0.506) was more negative than in the

Abstain (β = -0.300; SE = 0.201; p < 0.001; q < 0.001), Low (β = -0.625; SE = 0.419; p = 0.002; q = 0.005), ModDisc (β = 0.058; SE = 0.419; p < 0.001; q < 0.001), and HighMod groups (β = -0.172; SE = 0.359; p < 0.001; q < 0.001) (Figure 4.2). All these differences remained significant after adjustment for multiple comparisons.

For female infants, the pattern was reversed. VHigh group (β = 1.546; SE = 0.584) showed a significantly more positive GPx-MDI relationship than both the Abstain (β = -0.059; SE = 0.219; p = 0.010; q = 0.039) and HighMod groups (β = -0.790; SE = 0.404; p = 0.001; q = 0.010), with both comparisons remaining significant after correction (Figure 4.2). HighMod group (β = -0.790; SE = 0.404) exhibited a more negative relationship than both the Low (β = 0.712; SE = 0.459; p = 0.014; q = 0.039) and ModDisc groups (β = 0.527; SE = 0.364; p = 0.016; q = 0.039).

Late Pregnancy Erythrocyte GPx Activity and 12-Month MDI Scores Analysis of the relationship between maternal GPx and infant mental development at 12 months showed significant interactions with both infant sex and maternal alcohol consumption patterns.

Sex-specific associations varied across alcohol trajectory groups. In the Abstain group, male infants (β = –0.572; SE = 0.349) showed a significantly more negative GPx-MDI relationship compared to female infants (β = 0.676; SE = 0.368; p = 0.014) (Figure 4.3). This sex difference was even more pronounced in the VHigh group, where males demonstrated a strong negative association (β = –1.989; SE = 0.730) while females exhibited a positive relationship (β = 1.458; SE = 0.953; p = 0.004).

Among male infants, the VHigh group (β = –1.989; SE = 0.730) displayed a more negative GPx-MDI relationship than both the ModDisc group (β = 0.241; SE = 0.687; p = 0.026; q = 0.132) and the HighMod group (β = 0.242; SE = 0.520; p = 0.012; q = 0.121) (Figure 4.3). These differences did not maintain significance after adjustment for multiple comparisons.

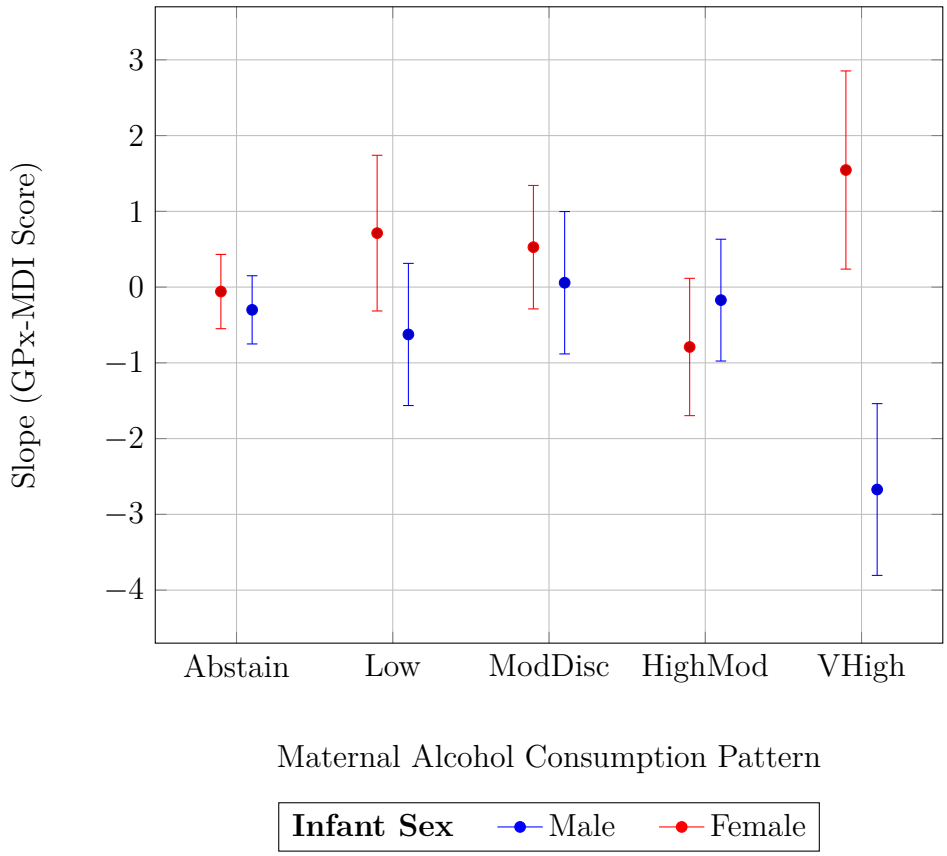

Figure 4.2: Association between maternal RBC GPx and infant 6-month MDI score by alcohol consumption pattern and infant sex

Late Pregnancy Erythrocyte GPx Activity and Infant Head Circumference at Birth Comparisons of the associations between maternal GPx and infant head circumference showed no significant sex differences within any of the alcohol trajectory groups, though a trend toward a sex difference was noted in the VHigh group (β = –0.325; SE = 0.171; p = 0.057) (Figure 4.4).

Among female infants, the VHigh trajectory group demonstrated a significantly stronger positive association between maternal GPx and infant head circumference (β = 0.386; SE = 0.119) compared to the Abstain group (β = 0.052; SE = 0.048; p = 0.009; q = 0.030), the Low group (β = –0.070; SE = 0.112; p = 0.005; q = 0.030), and the HighMod group (β =

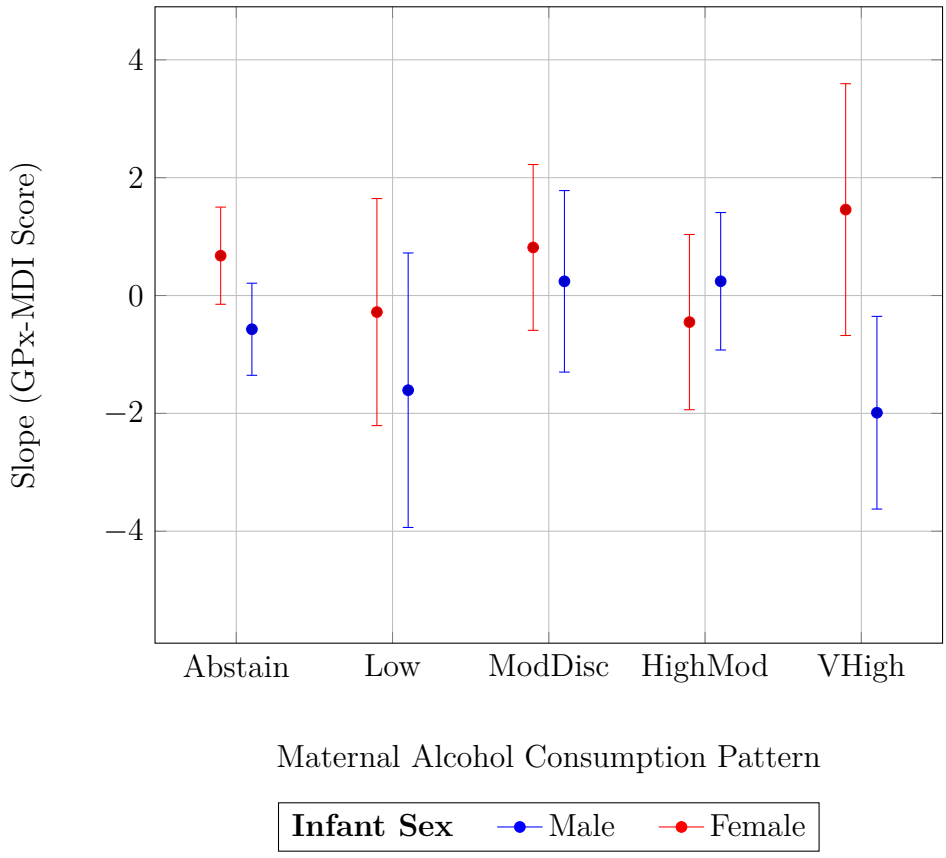

Figure 4.3: Association between maternal RBC GPx and infant 12-month MDI score by alcohol consumption pattern and infant sex

0.011; SE = 0.078; p = 0.008; q = 0.030) (Figure 4.4). A similar pattern was observed in comparison with the ModDisc group ($\beta = 0.074$; SE = 0.077; p = 0.028; q = 0.069), though this difference did not remain significant after adjustment for multiple comparisons.

For male infants, no significant differences in the GPx-head circumference relationship were observed across alcohol trajectory groups.

Early/Mid Pregnancy Erythrocyte SOD Activity and 6-Month PDI Scores Analysis of the relationship between maternal erythrocyte SOD and infant PDI scores at 6 months showed sex differences in the VHigh alcohol trajectory group, with female infants ($\beta =$

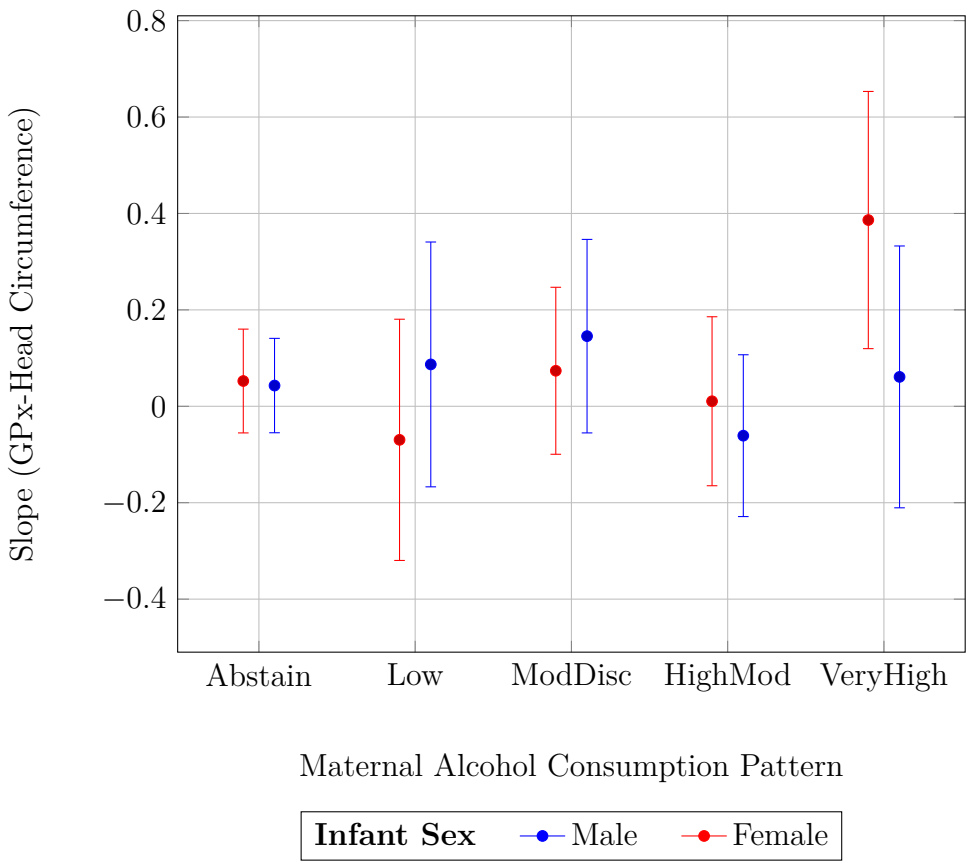

Figure 4.4: Association between maternal RBC GPx and infant head circumference by alcohol consumption pattern and infant sex

–0.058; SE = 0.027) demonstrating a more negative association between maternal SOD and PDI scores compared to male infants (β = 0.014; SE = 0.022; p = 0.038) (Figure 4.5). Within male and female infants, the relationship between maternal SOD and 6-month PDI scores did not differ significantly across alcohol trajectory groups.

Plasma Markers of Oxidative Stress and Antioxidant Capacity (TRAP and TBARS)

Late Pregnancy Plasma TRAP and 6-Month MDI Scores Analysis of maternal TRAP levels in relation to infant mental development at 6 months showed a notable sex-

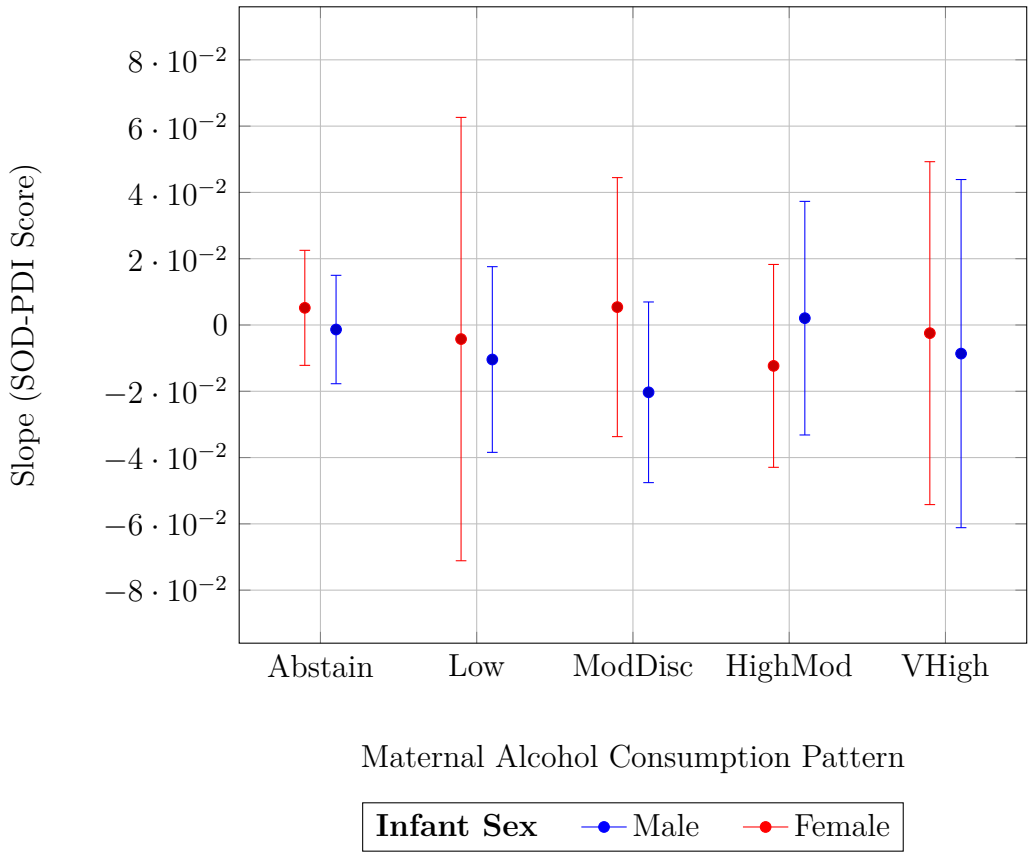

Figure 4.5: Association between maternal RBC SOD and infant 6-month PDI score by alcohol consumption pattern and infant sex

specific pattern in the HighMod alcohol group where male infants (β = –20.920; SE = 7.100) exhibited a significantly more negative association between maternal TRAP and infant MDI scores at 6 months compared to female infants (β = –0.770; SE = 6.250; p = 0.033) (Figure 4.6).

When examining differences across alcohol trajectory groups within each sex, male infants in the HighMod group (β = –20.920; SE = 7.100) showed a substantially more negative relationship between maternal TRAP and 6 month MDI score compared to males in the Abstain group (β = –1.150; SE = 2.540; p = 0.009; q = 0.090), though this difference only approached statistical significance after adjustment for multiple comparisons (Figure

4.6). Male infants in the HighMod group also demonstrated a more negative TRAP-MDI association than those in the VHigh group (β = –1.010; SE = 6.240; p = 0.035; q = 0.174), but this comparison did not retain significance after correction.

Among female infants, no significant differences across alcohol trajectory groups was observed.

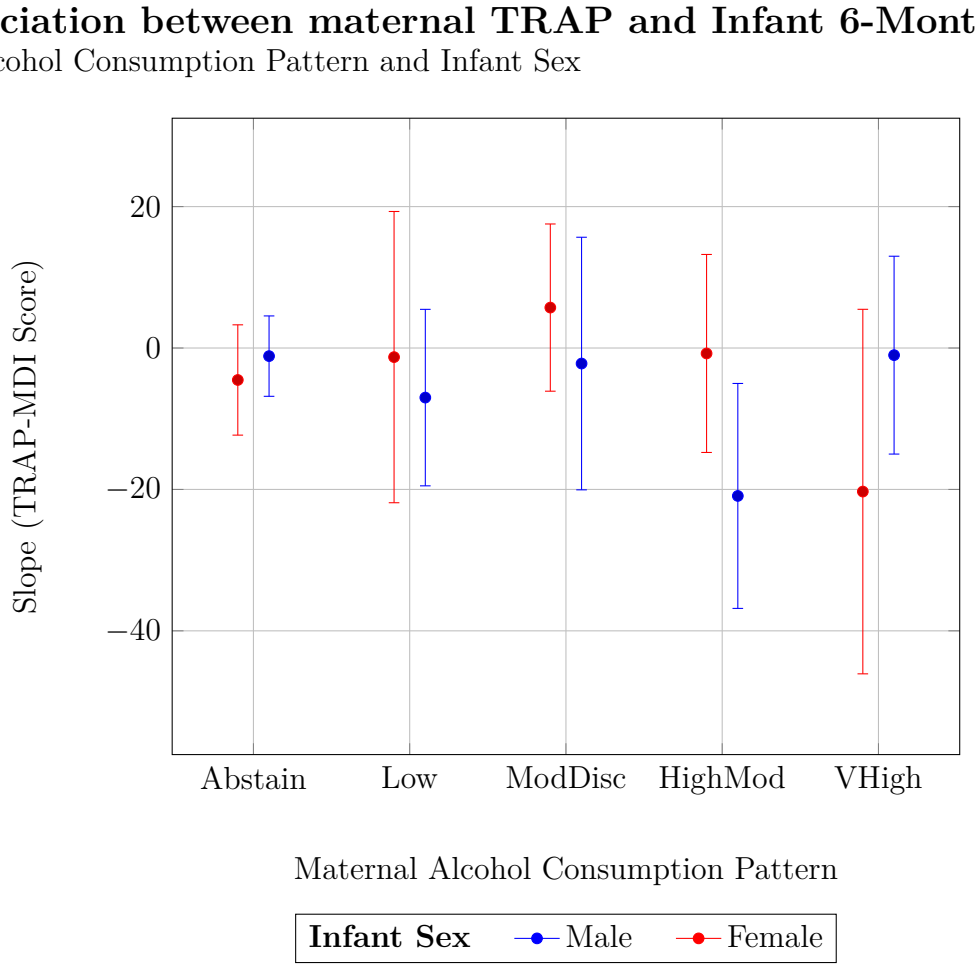

Figure 4.6: Association between maternal TRAP and infant 6-month MDI score by alcohol consumption pattern and infant sex

Early/Mid Pregnancy Plasma TBARS and 6-Month MDI Scores Analysis of maternal TBARS levels in relation to 6-month MDI score showed distinct patterns across infant sex and maternal alcohol trajectory groups. Sex differences emerged in the relationship

between maternal TBARS and 6-month MDI score within the ModDisc group (β = 16.589; SE = 6.366; p = 0.009), the HighMod group (β = –10.678; SE = 5.348; p = 0.046), and the VHigh group (β = 10.445; SE = 4.566; p = 0.022) (Figure 4.7).

Among male infants, those whose mothers followed the ModDisc trajectory exhibited a positive association between maternal TBARS and 6-month MDI scores (β = 16.724; SE = 5.660) (Figure 4.7). This relationship differed significantly from that observed in males from the Abstain group (β = –2.202; SE = 1.580; p = 0.001; q = 0.006), the Low group (β = –0.459; SE = 4.700; p = 0.019; q = 0.048), the HighMod group (β = –11.275; SE = 4.010; p < 0.001; q < 0.001), and the VHigh group (β = 2.160; SE = 3.810; p = 0.033; q = 0.059). The comparison with the VHigh group remained marginally significant after correction for multiple comparisons.

Male infants from the HighMod group showed a negative relationship between maternal TBARS and 6-month MDI score (β = –11.275; SE = 4.010) that differed from the Abstain group (β = –2.202; SE = 1.580; p = 0.035; q = 0.059), though this difference was marginally significant after adjustment (Figure 4.7). The relationship in this group also differed significantly from that observed in the VHigh group (β = 2.160; SE = 3.810; p = 0.016; q = 0.048).

Among female infants, no alcohol trajectory contrasts remained significant after adjusting for multiple comparisons.

Early/Mid Pregnancy Plasma TBARS and 6-Month PDI Scores Analysis of maternal TBARS levels showed significant associations with child developmental outcomes that varied by both infant sex and maternal alcohol consumption patterns.

Within the ModDisc alcohol trajectory group, male infants exhibited a markedly different relationship between maternal TBARS levels and 6-Month PDI compared to female infants (β = 33.377; SE = 11.261; p = 0.003) (Figure 4.8). Male infants showed a positive association (β = 36.420; SE = 10.200) while female infants demonstrated a negligible relationship (β =

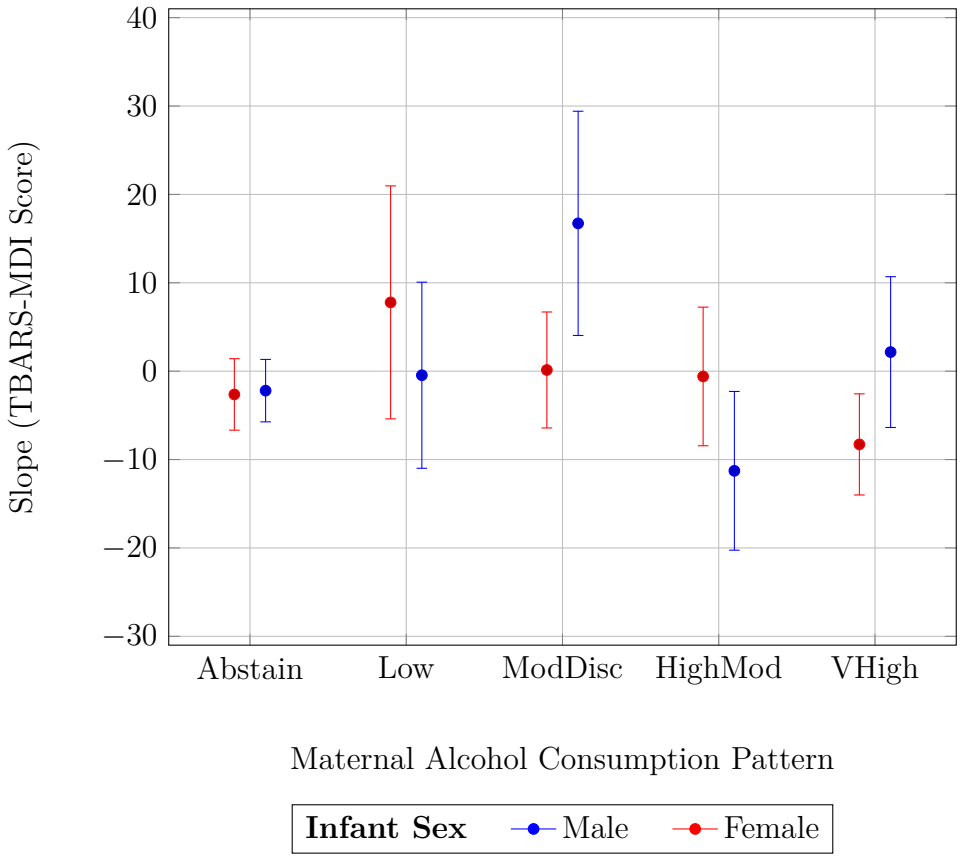

Figure 4.7: Association between maternal TBARS and infant 6-month MDI score by alcohol consumption pattern and infant sex

3.040; SE = 4.750).

Among male infants, maternal TBARS levels in the Low group demonstrated a strong positive association with 6-Month PDI (β = 24.880; SE = 8.790), which differed significantly from the relationship observed in the Abstain group (β = –5.340; SE = 2.600; p = 0.001; q = 0.002) (Figure 4.8). This pattern persisted when comparing the Low group to both the HighMod group (β = –7.940; SE = 6.800; p = 0.003; q = 0.006) and the VHigh group (β = –7.080; SE = 6.740; p = 0.004; q = 0.006).

The ModDisc group of male infants displayed the strongest positive association between maternal TBARS and 6-Month PDI (β = 36.420; SE = 10.200) (Figure 4.8). This rela-

tionship differed significantly from the patterns observed in the Abstain group (β = –5.340; SE = 2.600; p < 0.001; q < 0.001), the HighMod group (β = –7.940; SE = 6.800; p < 0.001; q = 0.001), and the VHigh group (β = –7.080; SE = 6.740; p < 0.001; q = 0.001). In contrast, female infants showed less pronounced differences across alcohol trajectory groups. The Low group (β = 14.080; SE = 7.490) had a stronger positive association between maternal TBARS and 6-Month PDI than both the Abstain group (β = –4.900; SE = 2.650; p = 0.017; q = 0.084) and the VHigh group (β = –6.560; SE = 4.010; p = 0.015; q = 0.084). These differences did not maintain statistical significance after adjustment for multiple comparisons.

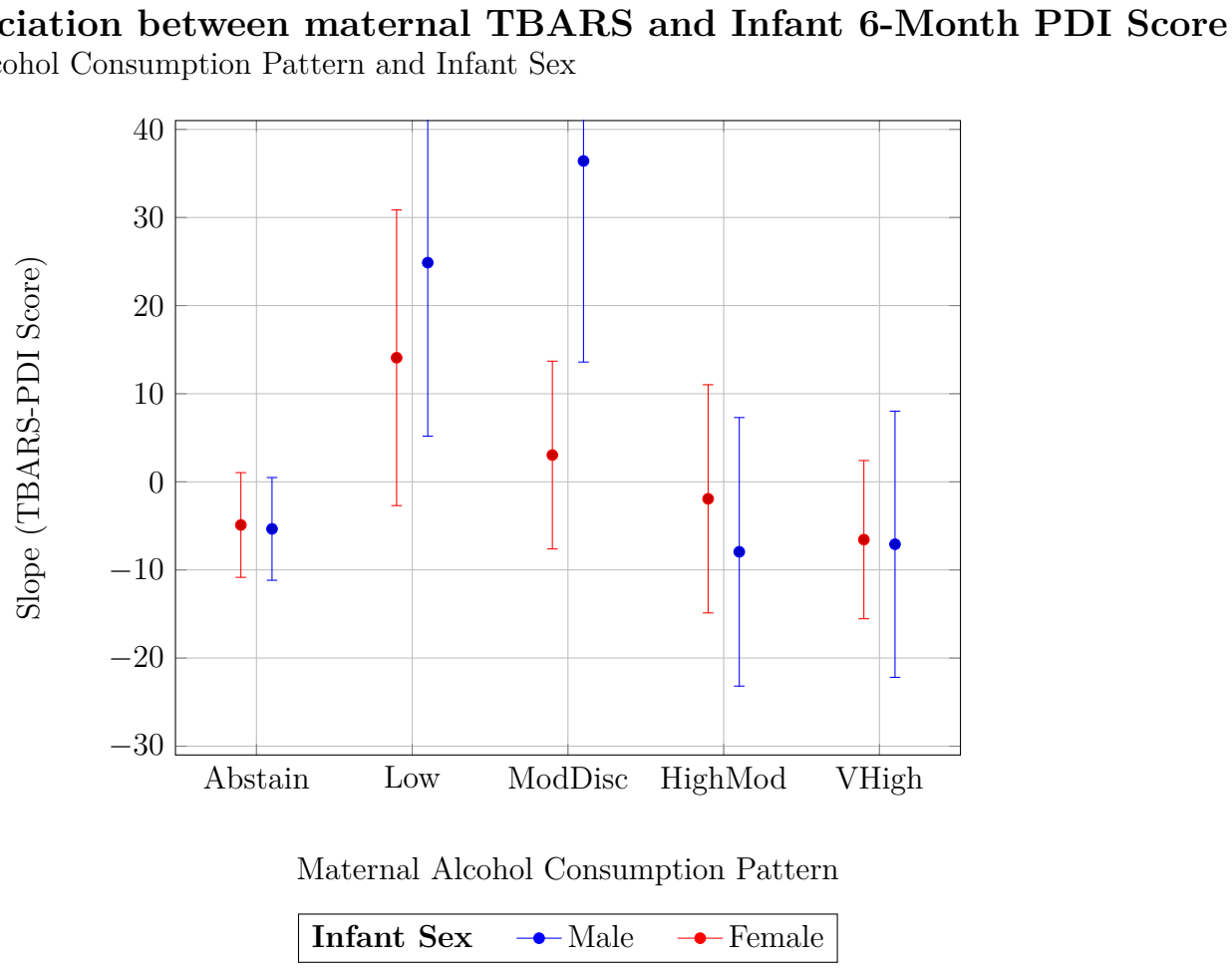

Figure 4.8: Association between maternal TBARS and infant 6-month PDI score by alcohol consumption pattern and infant sex

Markers of Inflammation and Related Micronutrients (hsCRP, CP, and Copper)

Early/Mid Pregnancy hsCRP and 12-Month MDI Scores Analysis of maternal high-sensitivity CRP in relation to infant MDI scores at 12 months showed no significant differences between male and female infants across all alcohol trajectory groups.

Among male infants, those born to mothers in the VHigh alcohol consumption group demonstrated a significant negative association between maternal hsCRP and 12-month MDI scores (β = -9.846; SE = 3.920) compared to all other trajectory groups (Figure 4.9). This negative relationship was significantly different from the positive associations observed in the ModDisc group (β = 5.194; SE = 3.720; p = 0.006; q = 0.028) and the HighMod group (β = 4.591; SE = 3.130; p = 0.004; q = 0.028), with both comparisons remaining significant after adjustment for multiple comparisons. The VHigh group also showed more negative hsCRP-MDI relationships than the Abstain group (β = -1.117; SE = 1.390; p = 0.036; q = 0.109) and the Low group (β = 1.913; SE = 4.290; p = 0.044; q = 0.109), though these differences did not retain significance after correction.

In contrast, female infants showed no significant differences in hsCRP-MDI relationships across alcohol trajectory groups.

Early/Mid Pregnancy hsCRP and Infant Head Circumference at Birth Analysis of the relationship between maternal hsCRP and infant head circumference showed significant sex differences across alcohol consumption trajectories.

Within the Low trajectory, male infants (β = -1.005; SE = 0.397) showed a significantly more negative relationship between maternal hsCRP levels and head circumference compared to female infants (β = 0.081; SE = 0.331; p = 0.036) (Figure 4.10). This pattern was more pronounced in the VHigh trajectory, where male infants exhibited a negative association (β = -0.829; SE = 0.406) while female infants demonstrated a positive association (β = 1.414; SE = 0.577; p = 0.001).

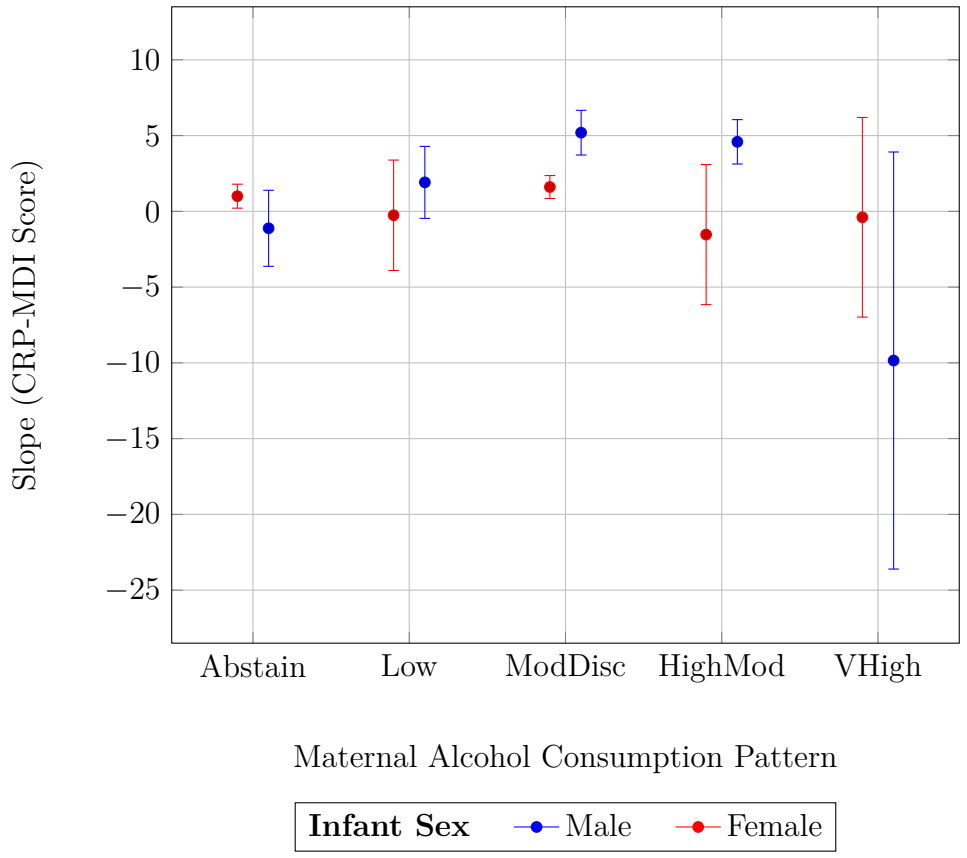

Figure 4.9: Association between maternal hsCRP and infant 12-month MDI score by alcohol consumption pattern and infant sex

Early/Mid Pregnancy Ceruloplasmin (CP) activity and Infant Head Circumference at Birth CP activity was related to infant head circumference in a pattern that varied by alcohol trajectory group and infant sex. In the VHigh trajectory group, females (β = 0.016; SE = 0.006) showed a significant positive relationship between maternal CP and head circumference, while males (β = -0.010; SE = 0.004) exhibited a significant negative association (β difference = -0.026; SE = 0.008; p = 0.001) (Figure 4.11).

Among female infants, those in the VHigh group demonstrated a significantly more positive CP-head circumference relationship than those in the ModDisc group (β = -0.005; SE = 0.004; β difference = -0.021; SE = 0.007; p = 0.004; q = 0.043), with this difference main-

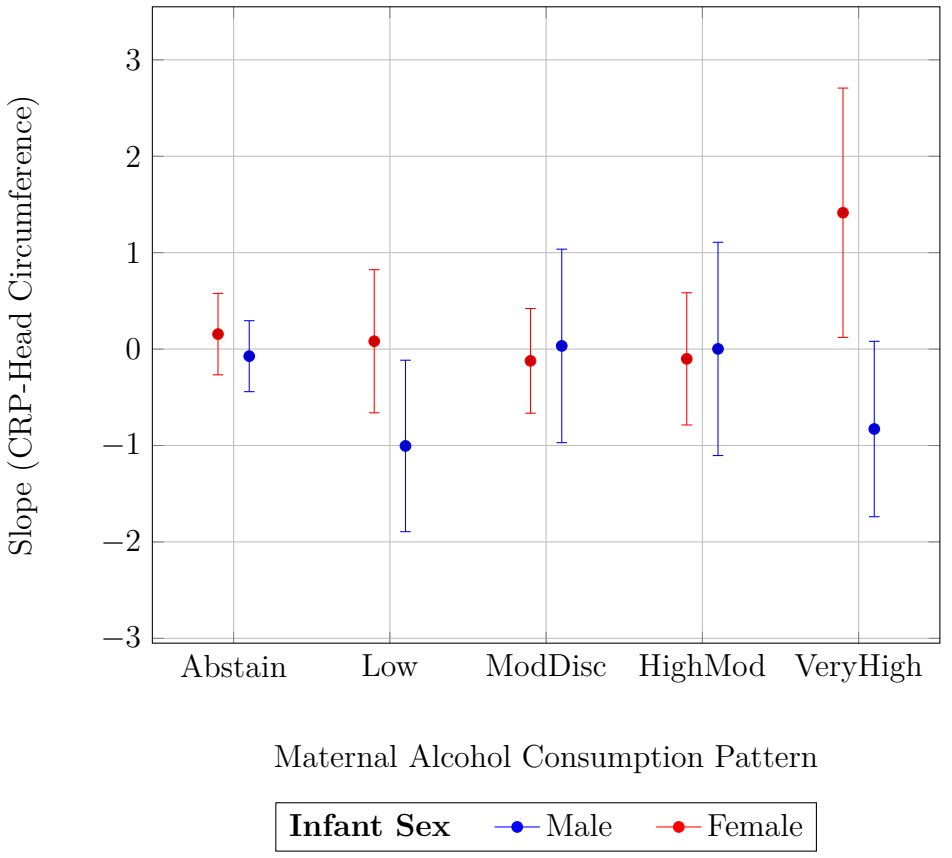

Figure 4.10: Association between maternal hsCRP and infant head circumference by alcohol consumption pattern and infant sex

taining significance after adjustment for multiple comparisons(Figure 4.11). VHigh group also showed marginally more positive relationships compared to the Abstain (β = 0.001; SE = 0.002; β difference = -0.014; SE = 0.007; p = 0.030; q = 0.080), Low (β = -0.001; SE = 0.004; β difference = -0.017; SE = 0.007; p = 0.021; q = 0.080), and HighMod groups (β = -0.001; SE = 0.005; β difference = -0.017; SE = 0.008; p = 0.032; q = 0.080), though these comparisons did not remain significant after adjustment.

For male infants, those in the VHigh group (β = -0.010; SE = 0.004) had a significantly more negative CP-head circumference relationship than the Abstain group (β = 0.003; SE = 0.002; β difference = 0.013; SE = 0.005; p = 0.005; q = 0.046), with this contrast remaining

significant after correction (Figure 4.11). Male infants in the Low (β = -0.006; SE = 0.004) and ModDisc groups (β = -0.008; SE = 0.005) also exhibited more negative relationships compared to the Abstain group (β differences = 0.010; SE = 0.005; p = 0.050; q = 0.165; and β difference = 0.011; SE = 0.005; p = 0.028; q = 0.139, respectively), though these differences did not maintain significance after adjustment.

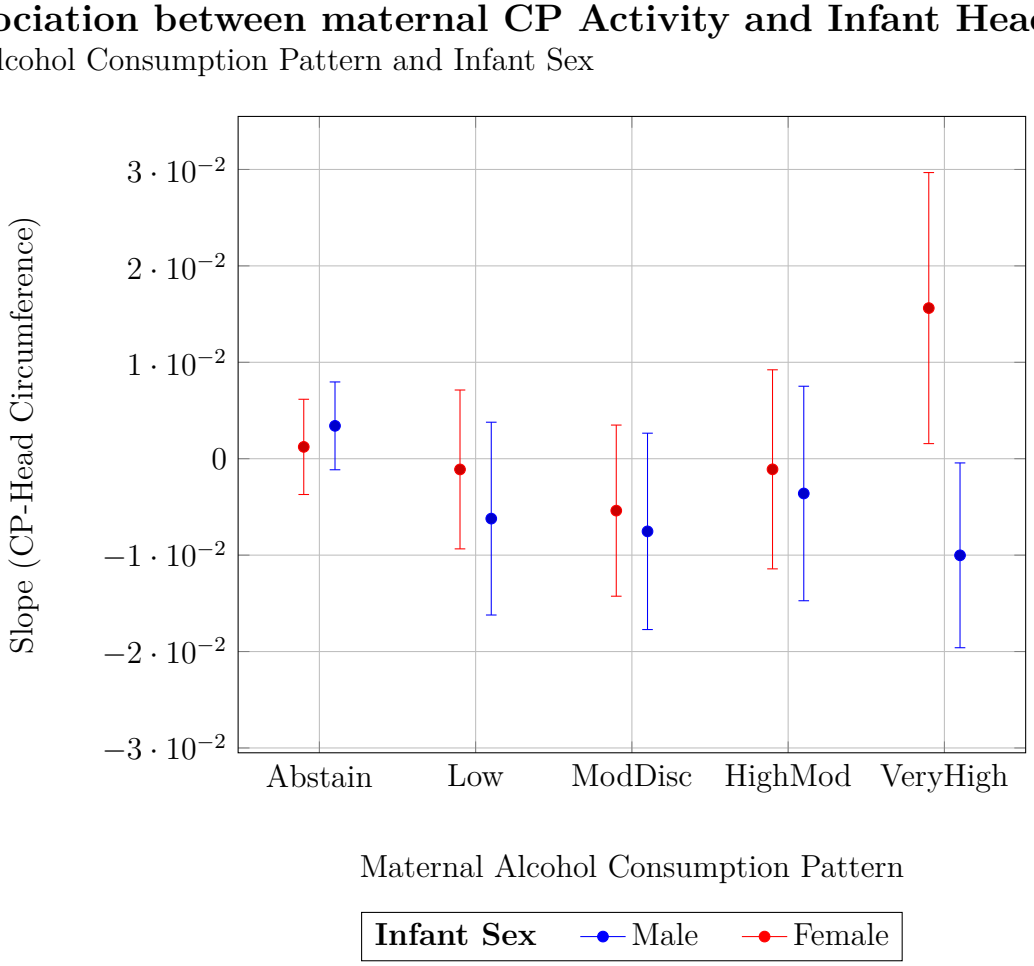

Figure 4.11: Association between maternal CP activity and infant head circumference by alcohol consumption pattern and infant sex

Early/Mid Pregnancy Plasma Copper and Infant Head Circumference at Birth

Analysis of the relationship between maternal plasma copper levels and infant birth head circumference showed a sex-specific pattern across alcohol consumption trajectories.

Within the VHigh alcohol trajectory group, male infants showed substantially smaller head circumferences in relation to maternal plasma copper levels compared to female infants (β = –4.254; SE = 1.447; p = 0.003) (Figure 4.12).

Males in the VHigh group demonstrated reduced head circumference measurements compared to males in the Abstain group (β = 3.678; SE = 1.345; p = 0.006; q = 0.062) (Figure 4.12). Similar patterns emerged when comparing the VHigh group to both the ModDisc group (β = 3.533; SE = 1.481; p = 0.017; q = 0.085) and the HighMod group (β = 3.245; SE = 1.534; p = 0.034; q = 0.115). These differences approached but did not maintain statistical significance after adjustment for multiple comparisons.

Female infants exhibited no significant differences in the relationship between maternal plasma copper levels and head circumference across alcohol trajectory groups.

Early/Mid Pregnancy Plasma Copper and Infant Length at Birth Maternal plasma copper concentrations exhibited varied associations with infant birth length across alcohol trajectory groups, with differences noted between male and female infants (Figure 4.13). Analysis showed a significant sex difference within the VHigh alcohol trajectory group (β = –6.012; SE = 2.325; p = 0.010). In this group, male infants showed a strong negative association between maternal plasma copper and birth length (β = –6.108; SE = 2.050), while female infants demonstrated a negligible relationship (β = –0.096; SE = 1.120).

Male infants born to mothers in the VHigh alcohol consumption group displayed a markedly negative relationship compared to those in the Abstain group (β = 6.524; SE = 2.109; p = 0.002; q = 0.020), the Low group (β = 6.761; SE = 2.705; p = 0.012; q = 0.047), and the HighMod group (β = 6.025; SE = 2.454; p = 0.014; q = 0.047) (Figure 4.13). These differences remained significant after adjustment for multiple comparisons. The contrast between the VHigh and ModDisc groups (β = 4.677; SE = 2.299; p = 0.042; q = 0.105) did not maintain significance after correction.

Female infants exhibited no significant differences in the relationship between maternal

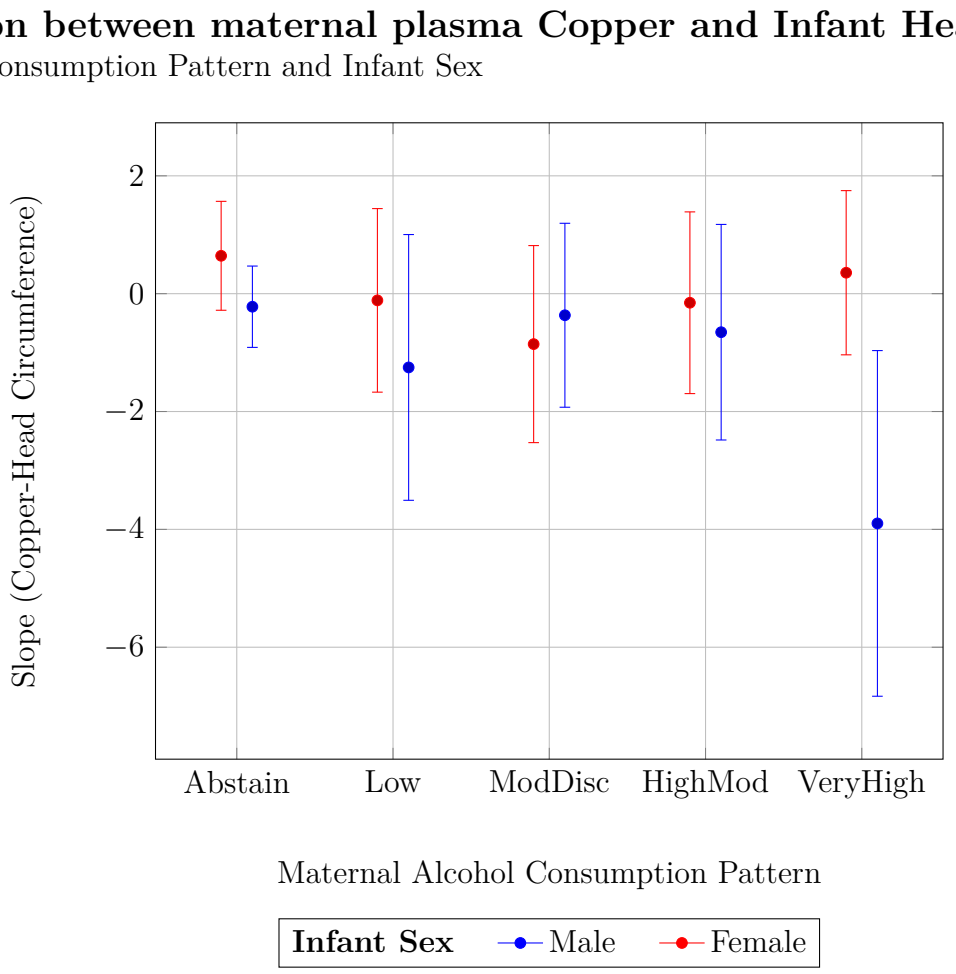

Figure 4.12: Association between maternal plasma copper and infant head circumference by alcohol consumption pattern and infant sex

plasma copper levels and length across alcohol trajectory groups.

Late Pregnancy Plasma Copper and 6-Month MDI Scores Analysis of associations between maternal plasma copper levels and infant MDI at 6 months showed distinct patterns across alcohol trajectory groups that varied by infant sex.

Sex comparisons showed that the relationship between maternal plasma copper and MDI scores differed significantly between male and female infants in two alcohol trajectory groups. In the Low group, female infants displayed a negative association (β = –6.963; SE = 4.430) compared to the positive association observed in males (β = 5.550; SE = 3.950; p = 0.035)

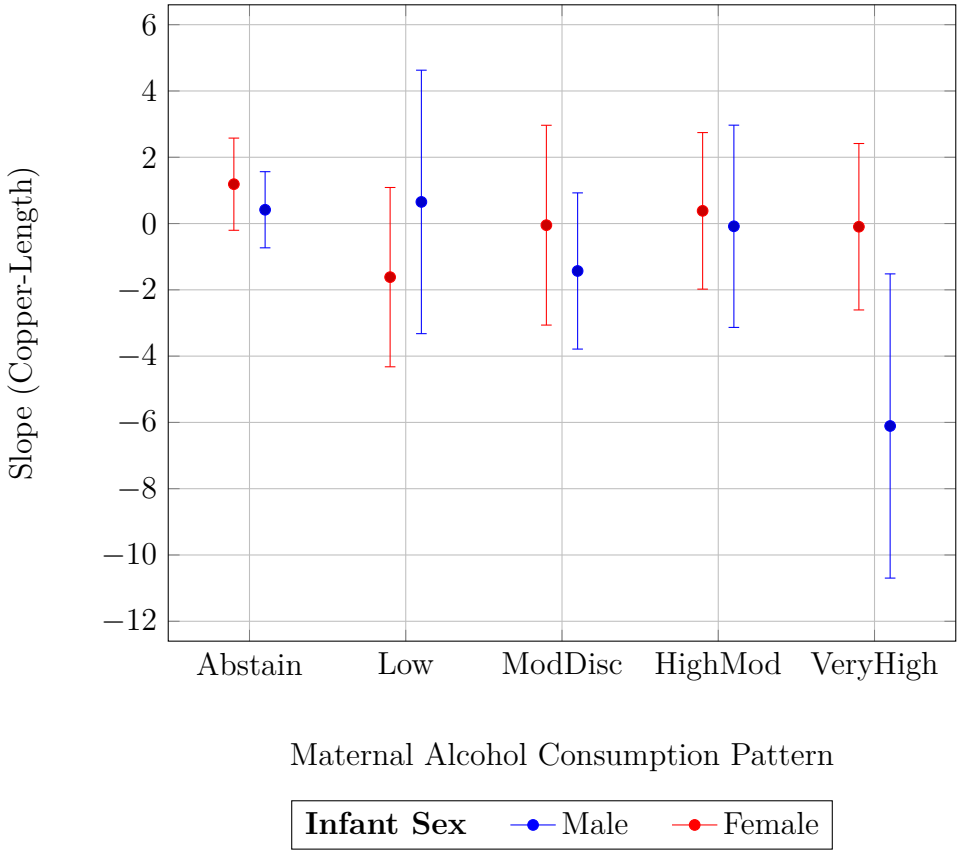

Figure 4.13: Association between maternal plasma copper and infant length by alcohol consumption pattern and infant sex

(Figure 4.14). This pattern was amplified in the VHigh group, where females exhibited a strong negative association (β = –23.089; SE = 6.290) while males showed a positive association (β = 9.358; SE = 10.200; p = 0.006).

Among female infants, the VHigh group demonstrated a negative association (β = –23.089; SE = 6.290), which differed significantly from the Abstain group (β = 1.197; SE = 1.440; p < 0.001; q = 0.002) (Figure 4.14). This negative association in the VHigh group also differed from the Low group (β = –6.963; SE = 4.430; p = 0.036; q = 0.091), though this comparison missed significance after multiple comparison adjustment. The VHigh group showed a significantly stronger negative association compared to both the ModDisc group

(β = 1.921; SE = 4.730; p = 0.002; q = 0.007) and the HighMod group (β = –1.864; SE = 3.350; p = 0.002; q = 0.007).

In contrast, male infants demonstrated no significant differences in the association between maternal plasma copper and MDI scores across alcohol trajectory groups.

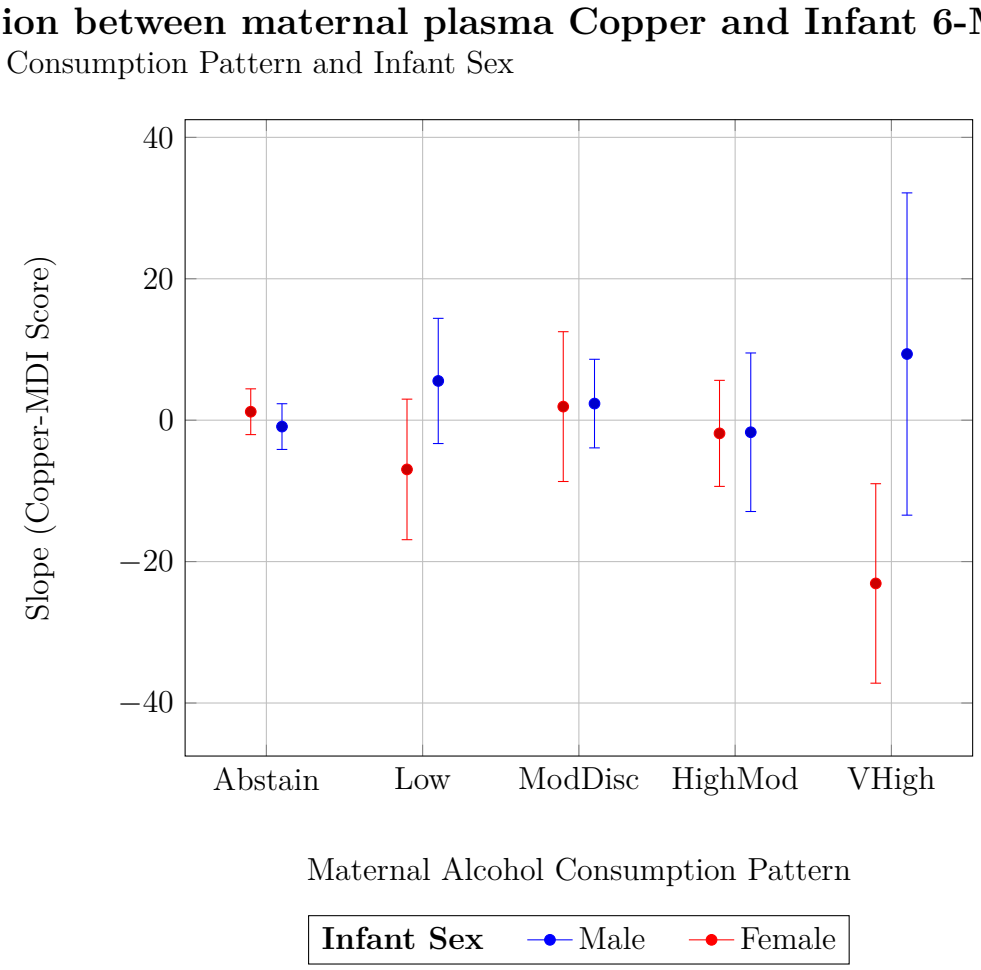

Figure 4.14: Association between maternal plasma copper and infant 6-month MDI score by alcohol consumption pattern and infant sex

Late Pregnancy Plasma Copper and 12-Month MDI Scores Analysis of the association between maternal plasma copper levels and infant MDI score at 12 months showed a significant sex-specific pattern within alcohol trajectory groups.

In the VHigh alcohol trajectory group, female infants (β = –19.960; SE = 8.220) exhibited

a strong negative relationship between maternal plasma copper and mental development, while male infants showed a positive relationship (β = 12.840; SE = 6.730), resulting in a significant sex difference (β difference = 32.808; SE = 10.706; p = 0.002) (Figure 4.15).

Among female infants, the VHigh group demonstrated a considerably more negative copper-MDI relationship compared to other trajectory groups (Figure 4.15). This negative association in the VHigh group differed from the positive relationships observed in the Abstain group (β = 2.000; SE = 2.590; p = 0.011; q = 0.055), the HighMod group (β = 6.350; SE = 5.280; p = 0.007; q = 0.055), and the ModDisc group (β = 7.210; SE = 8.240; p = 0.020; q = 0.066). These contrasts were marginally significant after multiple comparison adjustment.

Male infants showed no significant differences in the copper-MDI relationship across alcohol trajectory groups.

Late Pregnancy Plasma Copper and 12-Month PDI Scores Analysis of the relationship between maternal plasma copper levels and PDI at 12 months showed a significant sex difference within the VHigh alcohol trajectory group. Male infants displayed a positive association (β = 6.535; SE = 7.350) while female infants exhibited a strong negative relationship (β = -22.212; SE = 8.970; p = 0.014) (Figure 4.16).

Female infants in the VHigh group showed a significantly more negative copper-PDI relationship than females in the ModDisc group (β = 14.110; SE = 8.990; p = 0.004; q = 0.044) and the HighMod group (β = 3.651; SE = 5.760; p = 0.015; q = 0.075) (Figure 4.16). A similar pattern emerged when comparing the VHigh group to the Abstain group (β = -3.111; SE = 2.830; p = 0.042; q = 0.141), though this difference did not retain significance after multiple comparison adjustment. This pattern was not observed across males across their alcohol trajectory groups.

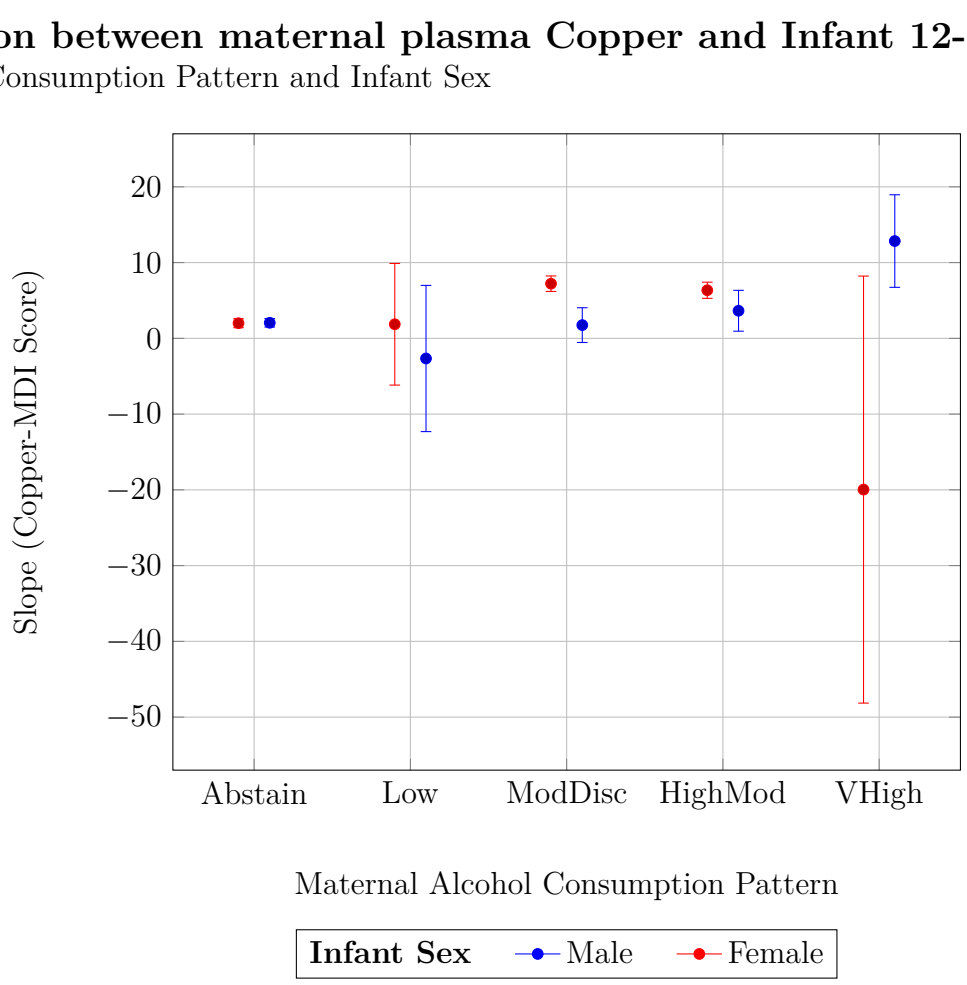

Figure 4.15: Association between maternal plasma copper and infant 12-month MDI score by alcohol consumption pattern and infant sex

4.4 Discussion

Results from Chapter 3 showed alcohol-induced alterations in maternal redox balance, APR, and nutrient homeostasis. The current work investigated connections between these maternal biochemical changes and infant development outcomes. We explored whether maternal biochemical and nutrient status, influenced by PAE, predicts infant growth and neurodevelopment, accounting for infant sex.

Our analysis identified three-way interactions among maternal biomarkers, alcohol exposure patterns, and infant sex. These interactions show that the relationship between

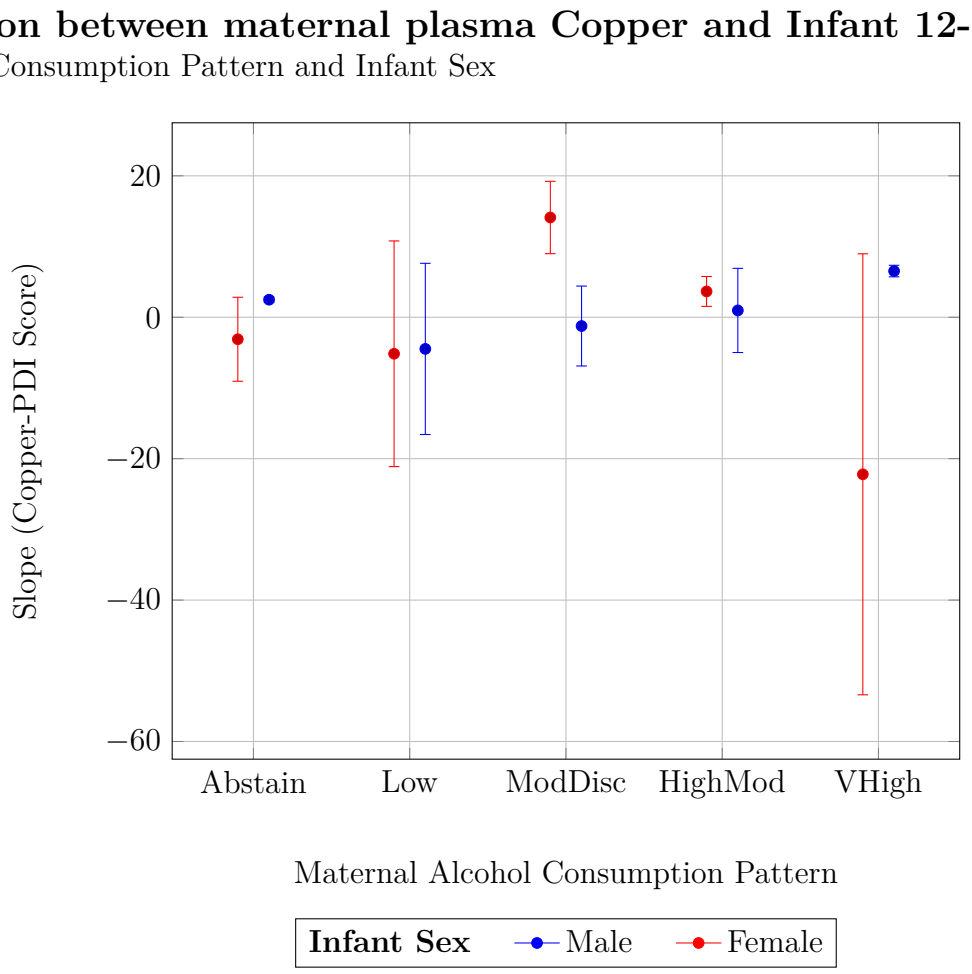

Figure 4.16: Association between maternal plasma copper and infant 12-month PDI score by alcohol consumption pattern and infant sex

maternal biochemical markers and child outcomes depends on the pattern and amount of PAE, and on infant sex. Many of the biomarkers we examined showed sex-differentiated responses. Within identical alcohol exposure groups, male and female infants exhibited distinct, sometimes divergent, associations between maternal biochemistry and developmental outcomes. This sex-differentiated pattern appeared for numerous biomarkers, including CP, hsCRP, and plasma copper.

The VHigh alcohol trajectory group presented biomarker-outcome connections distinct from other alcohol exposure patterns. This observation suggests a threshold effect where sustained high alcohol exposure disrupts normal physiological relationships. These findings

are consistent with observations in Chapter 3, which reported that this highest alcohol exposure group showed alterations in oxidative stress markers, inflammation indicators, and micronutrient status.

The timing of biomarker measurement was an important factor. Associations between maternal biochemistry and child outcomes varied with the timing of measurements during pregnancy. Maternal oxidative stress, inflammation, and nutrient dysregulation, influenced by alcohol consumption patterns throughout pregnancy, created distinct developmental environments. These environments affected male and female fetal and infant development differently. Maternal biochemical status seems to function as a mediating pathway between PAE and developmental outcomes. This pathway could offer intervention targets beyond alcohol abstinence.

4.4.1 Infant Outcomes and Prenatal Alcohol Exposure Trajectories

Infants born to mothers with a VHigh alcohol consumption trajectory exhibited poorer neurodevelopmental outcomes compared to infants in lower alcohol exposure groups, particularly the Abstain group. The VHigh group demonstrated lower MDI and PDI scores at both 6 and 12 months. Regarding birth outcomes, a trend towards lower birth weight was observed in the VHigh group relative to the Abstain group. Infant birth length showed a marginal association with alcohol trajectory group; pairwise comparisons did not achieve statistical significance, yet the VHigh group presented the smallest adjusted mean length. These findings are consistent with established teratogenic effects of PAE, which include impaired fetal growth and CNS disruption[10,12]. The pattern of poorer outcomes in the VHigh group points to a dose- and pattern-dependent effect of PAE. Sustained high levels of alcohol exposure appear linked to the greatest risk for adverse developmental outcomes[27,28]. Research employing similar longitudinal trajectory modeling of maternal drinking patterns within the same CIFASD cohort also found that a high, sustained PAE trajectory was associated with

deficits in infant weight and length at birth, and in psychomotor and mental performance at 6 to 12 months of age[27].

Maternal alcohol trajectory and infant sex interacted to influence certain outcomes. The observed deficits in neurodevelopmental scores (MDI and PDI at both 6 and 12 months) and the reduction in infant head circumference associated with the VHigh trajectory were predominantly evident in female infants. The trend for lower birth weight in the VHigh group compared to the Abstain group approached marginal significance only among females. Male infants in this study did not exhibit differences in these neurodevelopmental scores or birth weight across alcohol trajectory groups. Previous studies have reported sex-specific vulnerabilities to PAE. For example, Coles et al. (2015), in a study involving a partially overlapping CIFASD sample, found that higher periconceptual alcohol exposure more negatively affected MDI scores in male infants at 6 months[52]. That study also noted that MVM supplementation had a protective effect on MDI scores[52].

4.4.2 Maternal Acute Phase Response

The current study's observations point to a connection between PAE, maternal nutritional status, the APR, and infant development. These results are consistent with the perspective advanced by Keen et al. (2010), which posits that suboptimal maternal nutrition can intensify the teratogenicity of PAE, extending beyond the direct effects of nutrient deficiency or PAE alone[19]. A poor diet establishes a maternal-fetal environment where exposures, such as alcohol, exert magnified detrimental effects.

PAE can induce this suboptimal nutritional state partly through the APR. Stressors, including toxicants like alcohol, trigger an APR that involves pro-inflammatory cytokines such as TNF-α[19]. A notable outcome of this response is the stimulation of hepatic MT synthesis[24]. MT binds zinc, sequestering it in the maternal liver. This sequestration process can lead to maternal hypozincemia and diminish zinc transfer to the conceptus[23]. Experimental data corroborate this mechanism. Various toxicants, alcohol among them, induced

APR-linked reductions in fetal zinc uptake[24]. Administration of TNF-α replicated this effect, increasing maternal liver MT and zinc concentrations while concurrently decreasing plasma and embryonic zinc levels[25]. The teratogenicity of alcohol appeared reduced in MT-knockout mice, which are presumed to maintain more effective zinc transfer[23]. Furthermore, the teratogenicity of TNF-α is worsened by low maternal dietary zinc and lessened by supplemental zinc, demonstrating a direct interaction between APR inducing stress and maternal zinc status[25]. Embryo culture studies confirmed that serum from TNF-α treated animals impaired development; adding zinc to the culture medium rescued this impairment[25].

Analysis of maternal zinc status in the current study is consistent with these APR related mechanisms. Women who followed a VHigh drinking trajectory exhibited lower plasma zinc concentrations compared to abstainers. Zinc deficiency impairs development through multiple pathways, including abnormal nucleic acid metabolism, reduced protein synthesis, altered cell signaling, and increased apoptosis among others[19,53]. Zinc deficiency also inhibits neuronal cell proliferation and reduces neural progenitor cell proliferation[54,55], and it triggers apoptosis[54,56]. Zinc supports antioxidant defense via CuZnSOD and by inhibiting redox-active metal reactions[19,57]. Taken together, research shows that diminished maternal zinc availability can heighten fetal susceptibility to PAE-induced oxidative stress and disrupt developmental signaling[58].

Maternal plasma copper levels were lower, or showed a downward trend, in the VHigh group compared to abstainers. A typical APR includes hypercupremia, driven by CP production[59]. The lower plasma copper observed in the high exposure group in this study could reflect distinct chronic alcohol effects. Chronic alcohol ingestion in miniature pigs produced significantly lower plasma copper concentrations compared to controls[60]. This finding from an animal model indicates that long-term alcohol exposure can alter copper homeostasis, potentially counteracting or modifying an APR driven increase in plasma copper. Copper is a requisite for development, serving as a cofactor for enzymes involved in energy metabolism, connective tissue formation, and antioxidant defense (CuZnSOD)[61,62]. Copper deficiency

is teratogenic, affecting the CNS and growth[61,63], and can induce oxidative stress, leading to protein nitration in developing tissues[64]. Lower copper status represents an additional compromising nutritional factor for fetuses in the VHigh group.

The negative associations observed between these maternal biomarkers and infant outcomes, particularly within the VHigh group, could represent the amplified teratogenicity described by Keen and colleagues[19]. Adverse outcomes might not originate from direct PAE toxicity or a single nutrient deficit alone. Instead, they could arise from PAE occurring within a suboptimal nutritional milieu. This milieu is characterized by reduced zinc and copper availability, potentially aggravated by a sustained APR, rendering the fetus more vulnerable. This interpretation is consistent with animal research that shows compromised maternal nutrition worsens the effects of PAE, and that the teratogenicity of APR inducing agents is amplified by marginal zinc diets[25]. The co-occurrence in this analysis of negative associations for APR markers (hsCRP, CP) and copper with adverse infant outcomes in specific VHigh subgroups offers support for this view in human studies.

Among male infants born to mothers in the VHigh group, early/mid pregnancy findings showed maternal CP negatively associated with head circumference, hsCRP negatively associated with 12-month MDI, and copper negatively associated with both head circumference and length. This pattern points to an interaction between the APR, compromised copper status, and poorer growth and development, occurring with lower maternal zinc. For female infants in the VHigh group during early/mid pregnancy, a different pattern was observed: maternal CP showed a positive association with head circumference, without corresponding negative associations for copper. This positive CP association might reflect an antioxidant function, differing from the pattern seen in males or later in females, suggesting a chronic APR was not present in these females at this earlier stage. Later in pregnancy, another pattern emerged among female infants in the VHigh group where maternal hsCRP was negatively associated with infant length, and copper was negatively associated with MDI/PDI, indicating a potential APR influence at that later time point in pregnancy.

These sex- and timing-specific results indicate that within the context of VHigh PAE, a combination of heightened inflammation, shown by hsCRP/CP, and compromised copper status, alongside lower zinc, is associated with poorer infant growth and neurodevelopment. This pattern supports the hypothesis that the detrimental effects of PAE are magnified within a compromised maternal physiological state characterized by both nutritional and inflammatory markers[19].

4.4.3 Maternal Antioxidant Activity: Sex-Specific Associations with Infant Outcomes Under PAE

The analysis identified sex-specific associations between maternal late pregnancy erythrocyte GPx activity and infant outcomes in the VHigh alcohol trajectory group.

For male infants from the VHigh group, higher maternal erythrocyte GPx activity correlated negatively with 6-month MDI scores. A similar negative correlation with 12-month MDI scores did not achieve statistical significance after adjustment for multiple comparisons. These results suggest that elevated maternal GPx late in gestation with VHigh PAE does not correspond to improved cognitive outcomes for male offspring. Increased GPx activity might represent a response to severe oxidative stress, potentially indicating an overwhelmed maternal antioxidant system.

For female infants in the VHigh group, higher maternal GPx activity demonstrated a positive association with 6-month MDI scores and a positive trend appeared for 12-month MDI scores. Elevated late pregnancy maternal GPx activity further correlated with larger infant birth head circumference in this female subgroup. These relationships suggest higher maternal GPx activity relates to better 6-month cognitive development and growth in female offspring experiencing VHigh PAE. This finding points to a potential protective factor or a more resilient maternal antioxidant response with high PAE.

These sex-specific GPx relationships are consistent with the observation from late pregnancy erythrocyte GPx activity which was significantly higher in mothers carrying female

fetuses compared to male fetuses within the VHigh group. This baseline difference in maternal GPx activity between mothers of male and female infants in the highest alcohol exposure group may contribute to the divergent outcome associations. Such a difference potentially reflects underlying sex distinctions in antioxidant defenses or responses to sustained high levels of PAE.

Erythrocyte GPx, a selenium-dependent enzyme, neutralizes hydrogen peroxide. Its action is downstream of SOD, an enzyme that converts superoxide anions to hydrogen peroxide. GRed completes this antioxidant detoxification pathway by recycling the glutathione cofactor GPx requires. The distinct roles within this pathway offer an explanation for GPx activity, which represents the final hydrogen peroxide neutralization, correlating with outcomes, whereas SOD activity does not show a consistent correlation. Maternal late-pregnancy SOD activity was higher in the VHigh group carrying females compared to other exposure groups, including abstainers. This difference was marginal, losing statistical significance after adjustment for multiple comparisons. The absence of a strong outcome correlation for SOD is potentially attributable to its production of hydrogen peroxide, an intermediate ROS. This contrasts with GPx, which performs the final detoxification.

Interpreting maternal antioxidant status in the context of PAE presents complexities, evident in findings for plasma TRAP. Higher maternal TRAP levels during late gestation in the VHigh PAE group correlated negatively with 6-month PDI scores in female infants. This observation suggests that under conditions of very high PAE, elevated maternal TRAP (a measure of combined plasma antioxidant potential[65]) reflects a response to severe oxidative stress, not effective neuroprotection. This interpretation aligns with observations of increased TRAP accompanying oxidative stress from tobacco smoke[66]. Systemic TRAP perhaps does not represent defenses critical for organ protection, as these defenses are potentially intracellular or localized[67]. The negative TRAP-PDI correlation in females from the VHigh group, when contrasted with the potentially protective role of erythrocyte GPx in the same subgroup, indicates that different antioxidant markers can capture distinct maternal

physiological responses to PAE and can have differing impacts on the offspring.

4.4.4 Maternal Lipid Peroxidation and Infant Neurodevelopment

Analysis of maternal TBARS during early to mid-pregnancy showed sex-specific correlations with infant neurodevelopment at six months. In male infants, higher maternal TBARS levels correlated with improved MDI and PDI scores when PAE occurred in the Low or ModDisc trajectory groups. TBARS are conventionally understood as markers of lipid peroxidation and oxidative damage. The positive correlations observed in specific male infant groups could indicate that if these lipid peroxidation products signify damage, such damage is primarily maternal and not fetal.

The interpretation of maternal TBARS appears to differ by the level of PAE. With low to moderate PAE, TBARS may reflect adaptive maternal lipid metabolism rather than direct oxidative damage, explaining the positive neurodevelopmental correlations in males. Studies by Zidenberg-Cherr et al. gave a nuanced interpretation. In pigs fed ethanol chronically, hepatic lipid peroxidation assessed by TBARS was lower or not elevated compared to controls, despite antioxidant depletion. They hypothesized this resulted from chronic alcohol exposure having already peroxidized membrane polyunsaturated fatty acids before TBARS measurement. The depletion of highly unsaturated fatty acids, primary substrates for lipid peroxidation reactions, meant fewer substrates remained for further peroxidation. Such changes could render TBARS as inaccurate markers of peroxidative damage[60,68].

At higher PAE levels, alcohol's direct toxicity and increased ROS likely overwhelm any such adaptive responses. TBARS would then signify direct oxidative damage. The absence of these TBARS outcome correlations in female infants indicates sex specific differences in maternal or fetal responses to PAE induced alterations in lipid metabolism or oxidative stress.

4.4.5 Sex-Specific Associations Between Maternal Biomarkers and Infant Outcomes

This study observed interactions among maternal alcohol consumption trajectory, infant sex, and maternal biomarkers in predicting infant neurodevelopmental and anthropometric outcomes. The VHigh alcohol trajectory correlated with poorer neurodevelopment, lower birthweight, and smaller head circumference compared to abstainers; these effects were most apparent in female infants. Male infants exhibited fewer pronounced differences across alcohol trajectory groups for these outcomes. This observation concurs with human population studies indicating females may experience more severe neurobehavioral effects or dysmorphia after heavy PAE; reduced viability in heavily exposed males could contribute to this pattern as well[69,70]. Other research, often from clinical samples or animal models, suggests males experience greater neurodevelopmental impairment[70,71]. The findings present a complex picture where alcohol intake trajectory effects appeared stronger in females, yet associations between specific maternal physiological markers and infant outcomes were distinctly sex-dependent within the highest exposure VHigh group.

Fetal biological sex shapes the maternal physiological environment during pregnancy. Studies indicate fetal sex influences maternal immune profiles, including circulating cytokine levels and microRNA expression[70,72]. PAE also alters these maternal systems[70,72,73]. The sex-specific biomarker-outcome associations observed in our study may reflect the interplay between PAE-induced changes previously observed (Chapter 3) and this sex-influenced maternal milieu. For instance, Salem et al. (2020) reported that PAE-associated changes in maternal miRNA correlation networks were more pronounced or occurred earlier in pregnancies carrying female fetuses[72]. Our findings, such as the differing associations of maternal hsCRP, copper, or GPx with infant outcomes contingent on infant sex within the VHigh group, may stem from these underlying sex differences in maternal systemic responses to PAE.

Placental function offers another explanatory dimension. The placenta exhibits sex spe-

cific gene expression and adapts differently to prenatal stressors based on fetal sex[74–78]. The Trivers-Willard hypothesis posits that female placentas may prioritize adaptation under adverse conditions, while male placentas might prioritize growth. This distinction could lead to different vulnerabilities and compensatory responses when faced with PAE induced oxidative stress or changes in nutrient status[74,79]. Such differential placental strategies could mediate the sex-specific relationships we observed between maternal nutritional status or oxidative stress markers and fetal growth or neurodevelopment.

Distinct hormonal environments of male and female pregnancies may also contribute to these findings. Male fetuses produce testosterone, creating a different endocrine context compared to female fetuses[77,78]. PAE affects gonadal hormones and subsequent neurodevelopment in a sex dependent manner[80,81]. These hormonal differences could modulate maternal or placental responses to PAE. Androgens can influence pathways related to oxidative stress[82,83] and inflammation, including the acute phase response[77]. This interaction offers a potential mechanism for the observed sex differences in how markers like hsCRP, CP, or antioxidant enzymes relate to infant outcomes following high PAE. For instance, the opposing associations of late-pregnancy maternal GPx activity with infant MDI scores in the VHigh group might reflect sex-specific differences in the maternal antioxidant system's capacity or response under sustained alcohol-induced stress, possibly influenced by the hormonal milieu.

These results collectively indicate PAE does not exert uniform effects. Infant sex modifies its consequences, potentially through complex interactions involving sex specific maternal physiological responses, placental adaptations, and hormonal factors. Understanding these sex dependent pathways is important. Future research should continue to explore these mechanisms, perhaps incorporating direct measures of hormones or more detailed immune profiling. Recognizing sex as a key biological variable aids in accurately assessing risk and developing appropriately targeted interventions for FASD.

4.4.6 Maternal Iron: Lack of Associations with Outcomes

Animal models indicated PAE disrupts iron homeostasis. Rodent studies demonstrated PAE led to fetal anemia and functional iron deficiency, even with apparently adequate maternal dietary iron[84–86]. This disruption often involved altered iron metabolism, characterized by increased maternal and fetal hepcidin expression and sequestration of iron in the maternal liver, which reduced its availability to the fetus[85]. Maternal iron deficiency exacerbated the adverse effects of PAE on fetal growth and neurodevelopment[86,87]. Gestational iron supplementation in some animal models mitigated certain PAE-induced deficits, like fetal anemia and, in some instances, partially normalized brain weight[84]. A recent report by Hasken et al. (2024) found maternal ferritin was inversely associated with infant head circumference centile among AE infants[88].

Our analysis of infant data did not identify significant interactions involving maternal iron status markers. Maternal plasma iron, ferritin, and TfR levels did not show significant three-way interactions or significant two-way interactions with any measured infant anthropometric or neurodevelopmental outcomes.

4.4.7 Strengths and Limitations

Using GMM to describe longitudinal PAE trajectories provided a detailed picture of exposure patterns, an improvement over binary classifications of alcohol use. The study linked these PAE trajectories with numerous maternal biochemical markers, indicating oxidative stress, antioxidant defense, and micronutrient status. This work extended prior research (Chapter 3) through a direct assessment of associations among these detailed PAE patterns, the associated maternal physiological state, and infant developmental outcomes, considering infant sex as a moderating factor. Collecting data in the Ukrainian research setting, a location where alcohol reporting may face less stigma compared to some other regions, potentially improved the accuracy of reported consumption.

Some limitations merit discussion. The findings may have restricted generalizability. The

Western Ukrainian cohort possesses specific attributes, such as the absence of folic acid food fortification[89,90], which could affect baseline nutritional status. The observed relationships might not directly apply to populations with different dietary habits, genetic backgrounds, or healthcare systems[52,91]. The sample, consisting mainly of individuals at increased risk for PAE, potentially influenced the identified consumption trajectories relative to lower-risk or general population samples[27]. Maternal self-report of alcohol consumption, acquired using a standardized TLFB method, introduces a potential source of bias. Systematic underreporting or recall inaccuracies, if connected to trajectory group membership, could alter the estimated associations[27]. Residual confounding from unmeasured variables, such as comprehensive maternal diet beyond supplement use, paternal characteristics, genetic variants impacting alcohol or nutrient metabolism, and other environmental exposures, remains possible, even with statistical adjustment for many potential confounders[27,91]. The lack of detailed dietary intake information[89] complicates assessing how pre-existing dietary patterns might have shaped the maternal biochemical environment.

The BSID-II scales, administered early in life, might not detect the full spectrum or long-term manifestations of PAE associated neurodevelopmental outcomes[27,91]. The intricate nature of nutrient metabolism and interactions indicates the interplay between PAE, maternal physiology, and infant sex is complex. Prudence is necessary when drawing firm conclusions about specific biomarker-outcome connections, especially with considerable alcohol exposure.

4.5 Conclusion

Distinct maternal PAE trajectories, maternal biochemical status, and infant sex complexly interact, affecting infant anthropometric and neurodevelopmental outcomes. The specific pattern of maternal alcohol consumption during pregnancy, not just the presence of alcohol exposure, acts as a critical determinant. This study presents human evidence of an alcohol-

induced APR, apparent in the VHigh exposure group, that associates with adverse infant outcomes. This observation supports Keen et al.'s hypothesis of PAE-induced conditional nutrient deficiency and magnified teratogenicity.

A combination of maternal APR markers (hsCRP, CP) with associated micronutrient dysregulation, including lower copper and lower zinc in the VHigh group, correlates with negative infant growth and neurodevelopment. As an example, for male infants in the VHigh alcohol trajectory, mothers with higher CP and hsCRP in early to mid-pregnancy, combined with lower plasma copper, was associated with poorer head circumference, length, and cognitive score. This pattern of altered maternal physiology points to a detrimental maternal environment where PAE's effects are amplified. Different outcomes appear when elevations in an isolated APR marker occurs. The positive association between early/mid-pregnancy maternal CP and head circumference in female infants from the VHigh alcohol group was surprising. It is possible that in these mothers, a distinct, less severe, or transient compensatory maternal response occurred, unlike the sustained detrimental impact observed when a broader network of APR disturbance occurs.

Infant sex modulated the correlation between these maternal physiological states, including the APR, to developmental consequences. Alterations in maternal systems under sustained high alcohol exposure associated with different, sometimes opposing, infant outcomes for males and females. To illustrate, maternal erythrocyte GPx activity during late pregnancy in the VHigh group correlated with infant neurodevelopment divergently for male and female infants. These sex differentiated responses highlight the necessity of infant sex as a biological variable in FASD research, especially when interpreting maternal physiological responses like the APR.

These results support nuanced risk assessment for PAE-related harm. This assessment extends beyond simple alcohol use classifications, incorporating alcohol exposure patterns and the mediating roles of maternal physiology, including the APR, and infant sex. The maternal biochemical environment, especially the inflammatory and nutrient-related aspects

of the APR, is potentially modifiable. This environment could represent a point for interventions to mitigate some adverse PAE consequences. Future research should delineate the mechanisms of the PAE-induced APR and its sex specific fetal impacts. Investigations require direct assessments of placental function, maternal and fetal hormonal profiles, and detailed immune system responses across varied PAE patterns. Examining these interactions in diverse populations is important for clarifying the generalizability of these findings and for developing more targeted, effective prevention and intervention strategies for FASD.

4.6 Acknowledgements

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Bibliography

[1] Svetlana Popova. "Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis". en. In: *The Lancet Global Health* 5.3 (2017), p. 10. DOI: 10.1016/S2214-109X(17)30021-9.

[2] D. Lemoine. "Les enfants de parents alcoholiques Anomalies, observees de 127 cas". In: *Quest. Medical* 25 (1968). 01275, pp. 477–482.

[3] Kenneth Lyons Jones and David W. Smith. "Recognition of the Fetal Alcohol Syndrome in Early Infancy". en. In: *The Lancet*. Originally published as Volume 2, Issue 7836 302.7836 (Nov. 1973). 03391, pp. 999–1001. DOI: 10.1016/s0140-6736(73)91092-1.

[4] Shannon Lange et al. "Global prevalence of fetal alcohol spectrum disorder among children and youth: a systematic review and meta-analysis". en. In: *Obstetrical & Gynecological Survey* 73.4 (Apr. 2018), pp. 189–191. DOI: 10.1097/01.ogx.0000532194.88210.00.

[5] Philip A. May et al. “Estimating the community prevalence, child traits, and maternal risk factors of fetal alcohol spectrum disorders (FASD) from a random sample of school children.” en. In: *Drug and Alcohol Dependence* 227 (2021). tex.litmapsid: 125570027 tex.pubmedid: 34388579, p. 108918. DOI: 10.1016/J.DRUGALCDEP.2021.108918.

[6] Philip A. May et al. “Fetal Alcohol Spectrum Disorders in a Rocky Mountain Region City: Child Characteristics, Maternal Risk Traits, and Prevalence”. en. In: *Alcoholism: Clinical and Experimental Research* 44.4 (2020), pp. 900–918. DOI: 10.1111/acer.14315.

[7] Philip A. May et al. “Fetal Alcohol Spectrum Disorders in a Midwestern City: Child Characteristics, Maternal Risk Traits, and Prevalence”. en. In: *Alcoholism: Clinical and Experimental Research* 44.4 (2020). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/acer.14314, pp. 919–938. DOI: 10.1111/acer.14314.

[8] Philip A. May et al. “Prevalence of Fetal Alcohol Spectrum Disorders in 4 US Communities”. en. In: *JAMA* 319.5 (Feb. 2018). 00334, p. 474. DOI: 10.1001/jama.2017.21896.

[9] Philip A. May et al. “The prevalence of fetal alcohol spectrum disorders in rural communities in South Africa: a third regional sample of child characteristics and maternal risk factors”. en. In: *Alcoholism: Clinical and Experimental Research* 46.10 (Oct. 2022), pp. 1819–1836. DOI: 10.1111/acer.14922.

[10] Institute of Medicine et al. *Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention, and Treatment*. en. Pages: 4991. Washington, D.C.: National Academies Press, Apr. 1996. DOI: 10.17226/4991.

[11] Martha Wilson Jones and W. Thomas Bass. “Fetal alcohol syndrome”. en. In: *Neonatal Network* 22.3 (Jan. 2003), pp. 63–70. DOI: 10.1891/0730-0832.22.3.63.

[12] Courtney Caputo, Erin Wood, and Leila Jabbour. “Impact of fetal alcohol exposure on body systems: a systematic review”. en. In: *Birth Defects Research, Part C: Embryo Today–Reviews* 108.2 (June 2016), pp. 174–180. DOI: 10.1002/bdrc.21129.

[13] Raja A. S. Mukherjee and Neil Aiton, eds. *Prevention, Recognition and Management of Fetal Alcohol Spectrum Disorders*. en. 1st ed. Springer Cham, 2021. DOI: 10.1007/978-3-030-73966-9.

[14] Alicja Staszek et al. “The influence of FASD on psychiatric disorders: a literature review on the mechanisms relating fetal alcohol spectrum disorders to mental health conditions in adulthood”. en. In: *Journal of Education, Health and Sport* 79 (Mar. 2025), p. 57862. DOI: 10.12775/JEHS.2025.79.57862.

[15] Philip A May and J Phillip Gossage. “Maternal Risk Factors for Fetal Alcohol Spectrum Disorders”. en. In: *Alcohol Res Health* 34.1 (2011), pp. 15–26.

[16] Sylvia Roozen et al. “Systematic literature review on which maternal alcohol behaviours are related to fetal alcohol spectrum disorders (FASD)”. en. In: *BMJ Open* 8.12 (Dec. 2018), e022578. DOI: 10.1136/bmjopen-2018-022578.

[17] Svetlana Popova et al. “Alcohol’s Impact on the Fetus”. en. In: *Nutrients* 13.10 (Sept. 2021), p. 3452. DOI: 10.3390/nu13103452.

[18] Albert E. Chudley and Geoff G. Hicks, eds. *Fetal Alcohol Spectrum Disorder: Advances in Research and Practice*. en. 1st ed. Neuromethods. New York, NY: Humana, 2022. DOI: 10.1007/978-1-0716-2613-9.

[19] Carl L. Keen et al. "The plausibility of maternal nutritional status being a contributing factor to the risk for fetal alcohol spectrum disorders: the potential influence of zinc status as an example". en. In: *BioFactors* 36.2 (Mar. 2010), pp. 125–135. DOI: 10.1002/biof.89.

[20] Shama Bhatia et al. "Oxidative stress and DNA damage in the mechanism of fetal alcohol spectrum disorders". en. In: *Birth Defects Research* 111.12 (July 2019), pp. 714–748. DOI: 10.1002/bdr2.1509.

[21] Sergio Terracina et al. "The impact of oxidative stress on the epigenetics of fetal alcohol spectrum disorders". en. In: *Antioxidants* 13.4 (Mar. 2024), p. 410. DOI: 10.3390/antiox13040410.

[22] Peter G. Wells et al. "Fetal oxidative stress mechanisms of neurodevelopmental deficits and exacerbation by ethanol and methamphetamine". en. In: *im* 108.2 (June 2016). 00035, pp. 108–130. DOI: 10.1002/bdrc.21134.

[23] L. C. Carey et al. "Maternal ethanol exposure is associated with decreased plasma zinc and increased fetal abnormalities in normal but not metallothionein-null mice". en. In: *Alcoholism: Clinical and Experimental Research* 24.2 (Feb. 2000), pp. 213–219.

[24] Marie Weldon Taubeneck et al. "Altered maternal zinc metabolism following exposure to diverse developmental toxicants". en. In: *Reproductive Toxicology* 8.1 (Jan. 1994), pp. 25–40. DOI: 10.1016/0890-6238(94)90064-7.

[25] M. W. Taubeneck et al. "Tumor necrosis factor-alpha alters maternal and embryonic zinc metabolism and is developmentally toxic in mice". eng. In: *The Journal of Nutrition* 125.4 (Apr. 1995), pp. 908–919. DOI: 10.1093/jn/125.4.908.

[26] Luke C. Carey et al. "Zinc Supplementation at the Time of Ethanol Exposure Ameliorates Teratogenicity in Mice". en. In: *Alcoholism: Clinical and Experimental Research* 27.1 (Jan. 2003), pp. 107–110. DOI: 10.1111/j.1530-0277.2003.tb02729.x.

[27] Gretchen Bandoli et al. "Patterns of prenatal alcohol use that predict infant growth and development". en. In: *Pediatrics* 143.2 (Feb. 2019). <sub>PMCID: PMC6361345, e20182399. DOI: 10.1542/peds.2018-2399.

[28] Gretchen Bandoli et al. "Patterns of Prenatal Alcohol Exposure and Alcohol-Related Dysmorphic Features". en. In: *Alcoholism: Clinical and Experimental Research* 44.10 (Oct. 2020), pp. 2045–2052. DOI: 10.1111/acer.14430.

[29] Andrew D. Arenson et al. "Implementation of a shared data repository and common data dictionary for fetal alcohol spectrum disorders research". In: *Alcohol*. Special Issue on Fetal Alcohol Spectrum Disorders: Diagnosis and Intervention 44.7 (Nov. 2010), pp. 643–647. DOI: 10.1016/j.alcohol.2009.08.007.

[30] Sarah N. Mattson et al. "Collaborative initiative on fetal alcohol spectrum disorders: methodology of clinical projects". en. In: *Alcohol* 44.7-8 (Nov. 2010), pp. 635–641. DOI: 10.1016/j.alcohol.2009.08.005.

[31] K.D. Sowell et al. “Implications of altered maternal cytokine concentrations on infant outcomes in children with prenatal alcohol exposure”. en. In: *Alcohol* 68 (May 2018), pp. 49–58. DOI: 10.1016/j.alcohol.2017.08.006.

[32] L.C. Sobell and Mark Sobell. “Timeline follow-back: A technique for assessing self-reported alcohol consumption”. In: *Measuring Alcohol Consumption: Psychosocial and Biochemical Methods* (Jan. 1992), pp. 41–72.

[33] Linda K. Muthén and Bengt O. Muthén. *Mplus User’s Guide*. en. Apr. 2017.

[34] Joe M. McCord and Irwin Fridovich. “Superoxide Dismutase”. en. In: *Journal of Biological Chemistry* 244.22 (Nov. 1969), pp. 6049–6055. DOI: 10.1016/S0021-9258(18)63504-5.

[35] R. A. Lawrence and R. F. Burk. “Glutathione peroxidase activity in selenium-deficient rat liver”. en. In: *Biochemical and Biophysical Research Communications* 71.4 (Aug. 1976), pp. 952–958. DOI: 10.1016/0006-291x(76)90747-6.

[36] N. Agergaard and P. Thode Jensen. “Procedure for blood glutathione peroxidase determination in cattle and swine”. en. In: *Acta Veterinaria Scandinavica* 23.4 (Dec. 1982). Number: 4 Publisher: BioMed Central, pp. 515–527. DOI: 10.1186/BF03546770.

[37] M. B. Cohen and D. L. Duvel. “Characterization of the inhibition of glutathione reductase and the recovery of enzyme activity in exponentially growing murine leukemia (L1210) cells treated with 1,3-bis(2-chloroethyl)-1-nitrosourea”. en. In: *Biochemical Pharmacology* 37.17 (Sept. 1988), pp. 3317–3320. DOI: 10.1016/0006-2952(88)90645-4.

[38] K.M. Rogers and R.C. Augusteyn. “Glutathione reductase in normal and cataractous human lenses”. en. In: *Experimental Eye Research* 27.6 (Dec. 1978), pp. 719–721. DOI: 10.1016/0014-4835(78)90041-6.

[39] D. R. Janero. “Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury”. en. In: *Free Radical Biology & Medicine* 9.6 (1990), pp. 515–540. DOI: 10.1016/0891-5849(90)90131-2.

[40] Kunio Yagi. “Assay for blood plasma or serum”. en. In: *Methods in Enzymology*. Vol. 105. Oxygen Radicals in Biological Systems. Academic Press, Jan. 1984, pp. 328–331. DOI: 10.1016/S0076-6879(84)05042-4.

[41] Cesar G. Fraga, Brian E. Leibovitz, and Al L. Tappel. “Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes”. en. In: *Free Radical Biology & Medicine* 4.3 (Jan. 1988), pp. 155–161. DOI: 10.1016/0891-5849(88)90023-8.

[42] Eduardo Lissi et al. “Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements”. en. In: *Free Radical Biology & Medicine* 18.2 (Feb. 1995), pp. 153–158. DOI: 10.1016/0891-5849(94)00117-3.

[43] Janice F. Wang et al. “A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage”. en. In: *Journal of Nutrition* 130.8 (Aug. 2000), 2115S–2119S. DOI: 10.1093/jn/130.8.2115S.

[44] Karl H Schosinsky, H Peter Lehmann, and Myrton F Beeler. "Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride". en. In: *Clinical Chemistry* 20.12 (Dec. 1974), pp. 1556–1563. DOI: 10.1093/clinchem/20.12.1556.

[45] N Bayley. *Bayley scales of infant development (BSID-2)*. San Antonio, TX: The Psychological Corporation, 1993.

[46] Daniel J. Stekhoven and Peter Bühlmann. "MissForest—non-parametric missing value imputation for mixed-type data". In: *Bioinformatics* 28.1 (Jan. 2012), pp. 112–118. DOI: 10.1093/bioinformatics/btr597.

[47] R Core Team. *R: a language and environment for statistical computing*. en. manual. Vienna, Austria: R Foundation for Statistical Computing, 2021.

[48] Steven P. Millard. *EnvStats: an R package for environmental statistics*. en. 2nd ed. New York, NY: Springer New York, 2013.

[49] Mollie E. Brooks et al. "glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling". en. In: *R Journal* 9.2 (2017), p. 378. DOI: 10.32614/RJ-2017-066.

[50] Yoav Benjamini and Yosef Hochberg. "Controlling the false discovery rate: a practical and powerful approach to multiple testing". en. In: *Journal of the Royal Statistical Society: Series B (methodological)* 57.1 (Jan. 1995), pp. 289–300. DOI: 10.1111/j.2517-6161.1995.tb02031.x.

[51] Shinichi Nakagawa. "A farewell to bonferroni: the problems of low statistical power and publication bias". en. In: *Behavioral Ecology* 15.6 (Nov. 2004), pp. 1044–1045. DOI: 10.1093/beheco/arh107.

[52] Claire D. Coles et al. "Dose and Timing of Prenatal Alcohol Exposure and Maternal Nutritional Supplements: Developmental Effects on 6-Month-Old Infants". en. In: *Maternal and Child Health Journal* 19.12 (Dec. 2015), pp. 2605–2614. DOI: 10.1007/s10995-015-1779-x.

[53] Janet Y. Uriu-Adams and Carl L. Keen. "Zinc and reproduction: effects of zinc deficiency on prenatal and early postnatal development". en. In: *Birth Defects Research Part B-developmental and Reproductive Toxicology* 89.4 (Aug. 2010), pp. 313–325. DOI: 10.1002/bdrb.20264.

[54] Ana M. Adamo et al. "The role of zinc in the modulation of neuronal proliferation and apoptosis". eng. In: *Neurotoxicity Research* 17.1 (Jan. 2010), pp. 1–14. DOI: 10.1007/s12640-009-9067-4.

[55] Johnathan R. Nuttall et al. "Gestational marginal zinc deficiency impaired fetal neural progenitor cell proliferation by disrupting the ERK1/2 signaling pathway". en. In: *The Journal of Nutritional Biochemistry* 26.11 (Nov. 2015), pp. 1116–1123. DOI: 10.1016/j.jnutbio.2015.05.007.

[56] Huili Li, Jing Zhang, and Lee Niswander. "Zinc deficiency causes neural tube defects through attenuation of p53 ubiquitylation". en. In: *Development* 145.24 (Dec. 2018), dev169797. DOI: 10.1242/dev.169797.

[57] Patricia I. Oteiza. "Zinc and the modulation of redox homeostasis". en. In: *Free Radical Biology & Medicine* 53.9 (Nov. 2012), pp. 1748–1759. DOI: 10.1016/j.freeradbiomed.2012.08.568.

[58] Madeline M. Ross, Diego R. Hernandez-Espinosa, and Elias Aizenman. "Neurodevelopmental consequences of dietary zinc deficiency: a status report". en. In: *Biological Trace Element Research* 201.12 (Dec. 2023), pp. 5616–5639. DOI: 10.1007/s12011-023-03630-2.

[59] Carl L Keen et al. "Toxicant exposure and trace element metabolism in pregnancy". en. In: *Environmental Toxicology and Pharmacology* 4.3 (Dec. 1997), pp. 301–308. DOI: 10.1016/S1382-6689(97)10028-X.

[60] Sheri Zidenberg-Cherr et al. "The effect of chronic alcohol ingestion on free radical defense in the miniature pig". en. In: *Journal of Nutrition* 120.2 (Feb. 1990), pp. 213–217. DOI: 10.1093/jn/120.2.213.

[61] Cl Keen et al. "Effect of copper deficiency on prenatal development and pregnancy outcome". en. In: *The American Journal of Clinical Nutrition* 67.5 (May 1998), 1003S–1011S. DOI: 10.1093/ajcn/67.5.1003S.

[62] Janet Y. Uriu-Adams et al. "Influence of copper on early development: prenatal and postnatal considerations". en. In: *BioFactors* 36.2 (Mar. 2010), pp. 136–152. DOI: 10.1002/biof.85.

[63] Zivanit Ergaz et al. "The effect of copper deficiency on fetal growth and liver antioxidant capacity in the cohen diabetic rat model". en. In: *Toxicology and Applied Pharmacology* 265.2 (Dec. 2012), pp. 209–220. DOI: 10.1016/j.taap.2012.10.006.

[64] Molly E. Beckers-Trapp et al. "Abnormal development and increased 3-nitrotyrosine in copper-deficient mouse embryos". en. In: *Free Radical Biology and Medicine* 40.1 (Jan. 2006), pp. 35–44. DOI: 10.1016/j.freeradbiomed.2005.08.020.

[65] D.D.M. Wayner et al. "The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma". en. In: *Biochimica et Biophysica Acta (BBA) - General Subjects* 924.3 (June 1987), pp. 408–419. DOI: 10.1016/0304-4165(87)90155-3.

[66] L. Fayol et al. "Antioxidant Status of Neonates Exposed in utero to Tobacco Smoke". en. In: *Neonatology* 87.2 (2005), pp. 121–126. DOI: 10.1159/000082128.

[67] Josephine A. Drury et al. "Does Total Antioxidant Status Relate to Outcome in Very Preterm Infants?" en. In: *Clinical Science* 94.2 (Feb. 1998), pp. 197–201. DOI: 10.1042/cs0940197.

[68] Sheri Zidenberg-Cherr et al. "Ethanol-induced changes in hepatic free radical defense mechanisms and fatty-acid composition in the miniature pig". en-US. In: *Hepatology* 13.6 (June 1991), p. 1185. DOI: 10.1002/hep.1840130627.

[69] Philip A. May et al. "Who is most affected by prenatal alcohol exposure: boys or girls?" en. In: *Drug and Alcohol Dependence* 177 (Aug. 2017), pp. 258–267. DOI: 10.1016/j.drugalcdep.2017.04.010.

[70] Shameena Bake et al. “The interaction of genetic sex and prenatal alcohol exposure on health across the lifespan”. en. In: *Frontiers in Neuroendocrinology* 71 (Oct. 2023), p. 101103. DOI: 10.1016/j.yfrne.2023.101103.

[71] Katherine Flannigan et al. “Sex-related differences among individuals assessed for fetal alcohol spectrum disorder in Canada”. en. In: *Alcohol: Clinical and Experimental Research* 47.3 (Mar. 2023), pp. 613–623. DOI: 10.1111/acer.15017.

[72] Nihal A. Salem et al. “Association between fetal sex and maternal plasma microRNA responses to prenatal alcohol exposure: evidence from a birth outcome-stratified cohort”. en. In: *Biology of Sex Differences* 11.1 (Dec. 2020), p. 51. DOI: 10.1186/s13293-020-00327-2.

[73] Tamara S. Bodnar et al. “Altered maternal immune networks are associated with adverse child neurodevelopment: Impact of alcohol consumption during pregnancy”. en. In: *Brain, Behavior, and Immunity* 73 (Oct. 2018), pp. 205–215. DOI: 10.1016/j.bbi.2018.05.004.

[74] Sze Ting Cecilia Kwan et al. “An interaction between fetal sex and placental weight and efficiency predicts intrauterine growth in response to maternal protein insufficiency and gestational exposure window in a mouse model of FASD”. en. In: *Biology of Sex Differences* 11.1 (Dec. 2020), p. 40. DOI: 10.1186/s13293-020-00320-9.

[75] V.L. Clifton. “Review: sex and the human placenta: mediating differential strategies of fetal growth and survival”. en. In: *Placenta* 31 (Mar. 2010), S33–S39. DOI: 10.1016/j.placenta.2009.11.010.

[76] Ashley S. Meakin et al. “Let’s talk about placental sex, baby: understanding mechanisms that drive female- and Male-specific fetal growth and developmental outcomes”. en. In: *International journal of molecular sciences* 22.12 (Jan. 2021). Number: 12 Publisher: Multidisciplinary Digital Publishing Institute, p. 6386. DOI: 10.3390/ijms22126386.

[77] Kelly J Baines and Rachel C West. “Sex differences in innate and adaptive immunity impact fetal, placental, and maternal health”. en. In: *Biology of Reproduction* 109.3 (Sept. 2023), pp. 256–270. DOI: 10.1093/biolre/ioad072.

[78] Amy E. Braun et al. “Sex at the interface: the origin and impact of sex differences in the developing human placenta”. en. In: *Biology of Sex Differences* 13.1 (Sept. 2022), p. 50. DOI: 10.1186/s13293-022-00459-7.

[79] Robert L. Trivers and Dan E. Willard. “Natural selection of parental ability to vary the sex ratio of offspring”. en. In: *Science* 179.4068 (Jan. 1973). Publisher: American Association for the Advancement of Science, pp. 90–92. DOI: 10.1126/science.179.4068.90.

[80] K.A. Uban et al. “Sex differences in associations between white matter microstructure and gonadal hormones in children and adolescents with prenatal alcohol exposure”. en. In: *Psychoneuroendocrinology* 83 (Sept. 2017), pp. 111–121. DOI: 10.1016/j.psyneuen.2017.05.019.

[81] Amy L. Panczakiewicz et al. “Neurobehavioral Deficits Consistent Across Age and Sex in Youth with Prenatal Alcohol Exposure”. en. In: *Alcoholism: Clinical and Experimental Research* 40.9 (Sept. 2016), pp. 1971–1981. DOI: 10.1111/acer.13153.

[82] C. Massafra et al. “Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catalase and glutathione peroxidase activities during the menstrual cycle”. en. In: *Journal of Endocrinology* 167.3 (Dec. 2000). Publisher: BioScientifica Section: Journal of Endocrinology, pp. 447–452. DOI: 10.1677/joe.0.1670447.

[83] Marta Giergiel et al. “The influence of age and gender on antioxidant enzyme activities in humans and laboratory animals”. en. In: *Aging Clinical and Experimental Research* 24.6 (Dec. 2012), pp. 561–569. DOI: 10.1007/BF03654838.

[84] Kaylee K. Helfrich et al. “Gestational Iron Supplementation Improves Fetal Outcomes in a Rat Model of Prenatal Alcohol Exposure”. en. In: *Nutrients* 14.8 (Apr. 2022), p. 1653. DOI: 10.3390/nu14081653.

[85] Nipun Saini et al. “Alcohol’s Dysregulation of Maternal–Fetal IL-6 and p-STAT3 Is a Function of Maternal Iron Status”. en. In: *Alcoholism: Clinical and Experimental Research* 43.11 (Nov. 2019), pp. 2332–2343. DOI: 10.1111/acer.14200.

[86] Kaylee K. Helfrich et al. “Maternal iron nutriture as a critical modulator of fetal alcohol spectrum disorder risk in alcohol-exposed pregnancies”. en. In: *Biochemistry and Cell Biology* 96.2 (Apr. 2018), pp. 204–212. DOI: 10.1139/bcb-2017-0206.

[87] Echoleah S. Rufer et al. “Adequacy of maternal iron status protects against behavioral, neuroanatomical, and growth deficits in fetal alcohol spectrum disorders”. en. In: *PLoS ONE* 7.10 (Oct. 2012). Ed. by Jonathan I. Matsui, e47499. DOI: 10.1371/journal.pone.0047499.

[88] Julie M. Hasken et al. “Contribution of ferritin and zinc to adverse infant outcomes among pregnancies with prenatal alcohol exposure in South Africa”. en. In: *Reproductive Toxicology (Elmsford, N.Y.)* 127 (Aug. 2024), p. 108606. DOI: 10.1016/j.reprotox.2024.108606.

[89] J.A. Kable et al. “The impact of micronutrient supplementation in alcohol-exposed pregnancies on information processing skills in Ukrainian infants”. en. In: *Alcohol* 49.7 (Nov. 2015), pp. 647–656. DOI: 10.1016/j.alcohol.2015.08.005.

[90] J.A. Kable et al. “The impact of micronutrient supplementation in alcohol-exposed pregnancies on reaction time responses of preschoolers in Ukraine”. en. In: *Alcohol* 99 (Mar. 2022), pp. 49–58. DOI: 10.1016/j.alcohol.2021.12.002.

[91] Claire D. Coles et al. “Measurement of neurodevelopmental effects of prenatal alcohol exposure in Ukrainian preschool children”. en. In: *Child Neuropsychology* 27.8 (Nov. 2021), pp. 1088–1103. DOI: 10.1080/09297049.2021.1919298.

Chapter 5

Conclusion

The developmental harm from PAE is not uniform; it is dictated by the specific pattern of consumption, which creates a distinct maternal biochemical environment that is further modulated by infant sex. This dissertation moves beyond the simple classifications of 'drinker' versus 'non-drinker' by applying growth mixture modeling to identify these distinct longitudinal consumption patterns. By linking these data-driven trajectories to a comprehensive panel of maternal biochemical markers and subsequent infant outcomes, this work provides direct human evidence for the complex interplay between behavior, biology, and developmental vulnerability. The findings demonstrate that the pattern of drinking, not merely its presence, is a critical determinant of risk, enhancing the framework for understanding and potentially mitigating the impact of FASD.

The central finding is that a pattern of very high, sustained drinking throughout pregnancy creates a uniquely detrimental maternal environment. Women in this highest-exposure group exhibited physiological disruptions consistent with an alcohol-induced APR, including heightened inflammation, elevated oxidative stress, and depleted plasma zinc and copper. This maternal biochemical signature links a specific, high-risk drinking behavior to biological pathways that impair fetal development.

MVM supplementation during pregnancy partially mitigated these maternal disruptions.

Supplementation improved maternal antioxidant defenses and micronutrient status, even among the heaviest drinkers, suggesting nutritional support can bolster maternal physiological resilience against alcohol exposure. This maternal benefit did not translate into meaningful protection for the infant at the highest exposure levels. Infants born to mothers with a very high, sustained drinking trajectory had poorer outcomes, such as smaller head circumference and lower neurodevelopmental scores, regardless of maternal MVM use. A standard MVM supplement, beneficial for the mother, is insufficient to neutralize the severe neurodevelopmental consequences of very high PAE.

This research also highlights that infant sex is not a passive variable but an active modulator of risk. The findings suggest a bidirectional relationship, where male and female fetuses not only respond differently to the maternal environment created by PAE but may also influence that maternal physiological state itself. This was most apparent in the divergent, and sometimes opposing, associations between maternal biomarkers and infant outcomes. For example, in the highest-risk group, elevated maternal antioxidant activity correlated with better neurodevelopmental scores in females but poorer scores in males. These sex-specific dynamics are a critical factor for refining risk assessment and designing future research.

These findings have major implications for public health strategy and scientific inquiry. The results support moving beyond a uniform approach to PAE. Interventions must prioritize identifying and supporting women with very high, sustained drinking patterns, an extreme-risk group for whom standard nutritional advice is inadequate. These women require comprehensive strategies combining intensive alcohol cessation support with targeted interventions to address maternal inflammation and nutrient dysregulation.

This work generates hypotheses for future research. Its limitations, including variability in self-reported alcohol intake and non-standardized MVM supplementation, show the need for more rigorous study designs. Future studies should use frequent biological monitoring to validate alcohol consumption and standardized nutritional interventions with controlled timing and composition. Such work could test if targeted, high-dose supplementation of

specific nutrients like zinc offers greater fetal protection than a general MVM. Research must continue to explore the biological mechanisms of sex-specific vulnerability, such as placental adaptations and hormonal responses, to develop precise, sex-aware interventions.

This dissertation clarifies the pathway from specific PAE patterns to adverse infant outcomes. It demonstrates that maternal physiological state mediates alcohol's teratogenicity and that infant sex shapes the consequences. By identifying the highest-risk exposure patterns and revealing the limits of current nutritional support, this research builds a foundation for developing more effective, targeted strategies to prevent FASD.

Appendix A

Supplemental Code

```
MODEL:
  %OVERALL%
  i s | month01@0 month02@1 month03@2 month04@3 month05@4 month08@7
     month11@10;
  month08@0;
  %ALCINTAKE#1%
  i s;
  s WITH i@0;
  %ALCINTAKE#2%
  i s;
  s WITH i@0;
  %ALCINTAKE#3%
  i s;
  s WITH i@0;
  %ALCINTAKE#4%
  i s;
  s WITH i@0;
```

Listing A.1: Mplus Syntax for a GMM of Alcohol Consumption Trajectories