Maternal Prenatal Stress in Relation to Child Neurodevelopmental Outcome in MARBLES: A High Familial Risk Cohort

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Abstract

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by atypical development in social skills, language deficits, and restricted or repetitive interests and behaviors. In recent years, attention to ASD has increased due to the continual increase in prevalence. The CDC recently reported that 1 in 44 children in the United States was diagnosed with ASD in 2018, which is a nearly 23% increase since the reported estimate of 1 in 54 children just two years prior. It is unclear whether this increase is due to increasing awareness and improved detection or due to a true increase in the prevalence of this condition, as the etiology of ASD is still unknown.

The prenatal period is a sensitive time when a mother's behaviors and exposures can affect her child's long-term health. Like genetic factors, many prenatal environmental factors can have lasting effects on the neurodevelopment of the child. Identifying prenatal exposures and understanding how they influence ASD risk is an area of research that is developing but warrants more inquiry.

Maternal prenatal stress (MPS) is a complex exposure that depends on many factors, including the mother's financial standing, family events and complications, and ability to provide her family with basic needs. Associations between maternal MPS and behavioral differences in her offspring have been reported in experimental animal models using rodents and non-human primates. In human epidemiologic studies, estimated associations of maternal prenatal stress with ASD have varied findings. Some investigations indicate prenatal exposure to stress as being associated with risk of neurodevelopmental disorders, including Attention Deficit Hyperactivity Disorder and ASD. Three studies found increased risk of ASD in mothers exposed to MPS, measured by death of a first-degree relative, family discord, or broad recall of any stressful events. However, a few studies found no association with ASD when measuring MPS through: exposure to specific rocket attacks, prospective

collection of routine and major stressors, or experiencing death of a close relative. The discordant results might be explained by differing study designs. For example, the study examining death of a first-degree relative was retrospective, while the study examining death of a close relative was prospective. In addition, the studies' stress measurements varied from recall of very specific events, like rocket attacks, to very broad recall, such as asking an open-ended question of whether or not the mothers experienced any stressful events during pregnancy.

With inconsistent findings on the association between stressful life events and risk of ASD, this study measured stress in three ways and examined their respective associations with neurodevelopmental outcome (ASD, Typically developing (TD), or Non-typically developing (Non-TD)) in a high familial risk population, where pregnant women already had at least one other child with ASD so her subsequent children are at increased risk of developing ASD. In Chapter 1, the association between prenatal stressful life events and neurodevelopmental outcome was examined. In Chapter 2, the association of prenatal perceived stress and neurodevelopmental outcome was examined. In Chapter 3, the association of prenatal maternal urinary cortisol output and neurodevelopmental outcome was examined.

Findings indicated that generally, stressful life events were not associated with ASD and Non-TD outcome, though more research is needed to understand the increased relative risk of Non-TD (compared to TD) when experiencing legal problems, including immigration issues. Compared to TD, increased perceived stress was associated with higher relative risk of Non-TD in the first trimester, and with higher relative risk of ASD in the second and third trimester. Lastly, prenatal cortisol was not associated with neurodevelopmental outcome. These findings support existing literature showing that stressful life events are not associated with neurodevelopmental outcomes. Additionally, the increased risks of ASD seen in Chapter 2 in the second and third trimesters are also supported by previous investigations. Overall, this study's findings suggest that it may not be the stressful life events

experienced during pregnancy or how the mother's body biologically responds to these stressors, but instead how stressful the mothers perceive these events to be that may correlate with risk of ASD or Non-TD outcomes in the child. Stress reduction intervention could serve as preventative measures that help optimize the child's long term neurodevelopmental health in high familial risk families.

Dedication

To the memory of Elise Phelps Hanzel, who asked for so little but gave so very much.

Acknowledgements

Throughout my time in the graduate program, there were many people that never stopped supporting and believing in me. There were times when I became overwhelmed, but these individuals always saw the best in me and inspired me to keep persevering all the way through. They say it takes a village, and they definitely kept me motivated.

I would first like to thank my dissertation committee for their critical feedback that encouraged me to challenge myself in creating powerful research contributing to the growing field of autism research. Dr. Rebecca Schmidt trusted in me to work independently and pushed me to become a great problem solver. She always had helpful advice for those complex issues that arose. Dr. Daniel Tancredi always explained everything in such a digestible manner. He was so encouraging and somehow made SAS coding sound fun. He also engrained into my brain what the definition of a *p*-value is. Dr. Camelia Hostinar was such an invaluable resource. She would always promptly respond with some piece of existing literature that helped with my dissertation. She was an expert in her field but always remained so modest.

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My family is my backbone, and I could not have achieved such great successes if it were not for their unyielding support. My mother and father, being the caring Vietnamese parents they are, made sure I

was always nourished, and my sister and brother-in-law always picked up my slack of familial duties when I was under serious time crunches. Michelle, my best friend, was there to “go on walks” (we would walk by ourselves while talking on the phone with each other), proofread drafts, and just check on my level of sanity. James, my fiancé, made sure I always had what I needed and more. He always understood when I was under a lot of stress and never took anything personally. He just made sure to show up and reassure me.

Lastly, I would like to thank the late Dr. Elise Phelps Hanzel for entertaining my intellectual musings. She would always engage with me when I voiced my concerns about dissemination of research and public health application. She was always there to talk about whatever I needed, whether that be something personal, work-related, or school-related. My favorite thing was just letting her talk about her family or things she was passionate about, which allowed me to get my mind off of things. Elise made it a point to name me her “official academic public health connection” and believed that I could be one of the ones to move the needle in making good public health decisions. She lit a fire under me that kept me eager to do more.

I thank you all and cannot express enough how much I appreciate all of you.

Dorothy H. Hoang

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Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by atypical development in social skills, language deficits, and restricted or repetitive interests and behaviors.[1] In recent years, attention to ASD has increased due to the striking continual increase in prevalence. The CDC recently reported that 1 in 44 children in the United States was diagnosed with ASD in 2018, which is a nearly 23% increase since the reported estimate of 1 in 54 children just two years prior.[2] It is unclear whether this increase is due to increasing awareness and improved detection or due to a true increase in the prevalence of this disorder, as the etiology of the condition is still unknown.

The prenatal period is a sensitive time when a mother's behaviors and exposures can affect her child's long-term health.[3] Like genetic factors, many prenatal environmental factors can have lasting effects on the neurodevelopment of the child.[4-6] Identifying prenatal exposures and understanding how they influence ASD risk is an area of research that is developing but warrants more inquiry. Maternal prenatal stress (MPS) is a complex exposure that depends on many factors, including the mother's financial standing, family events and complications, and ability to provide her family with basic needs. Associations between maternal MPS and neurodevelopmental disorders in her offspring have been reported in previous studies.[7-20] Experimental animal models, using rodents and non-human primates, have found that induced MPS was associated with behavioral differences in the offspring, such as altered cardiovascular functioning, low birth weight, and delayed motor development.[7-10] However, difficulties of comparing rodent and non-human primate lifecycles to the human lifecycle make it hard to interpret these animal findings in the context of pregnant women. In human epidemiologic studies, estimated associations of maternal prenatal stress with ASD have varied. Some investigations indicate associations between prenatal exposure to stress and risk of neurodevelopmental disorders[11-15], including Attention Deficit Hyperactivity Disorder and ASD.[16-20] Three studies found increased risk of ASD in mothers exposed to MPS, measured by death of a first-degree relative,[21] family discord,[22] and broad

recall of any stressful events.[17,23] However, a few studies found no association with ASD when measuring MPS through: exposure to specific rocket attacks,[24] prospective collection of routine and major stressors,[25] or experiencing death of a close relative.[26] The discordant results might be explained by differing study designs. For example, the study examining death of a first-degree relative[21] was retrospective, while the study examining death of a close relative[26] was prospective. In addition, the studies' stress measurements varied from recall of very specific events, like rocket attacks, to very broad recall, such as asking an open-ended question of whether or not the mothers experienced any stressful events during pregnancy.

The studies exploring the association of stress in pregnant women and the risk of ASD are limited mainly to retrospective studies, and those that are prospective studies are limited in the type of stress event they measure. Additionally, maternal stress might be more of a concern for a child at high familial risk due to the mother already having a child diagnosed with ASD, meaning that she will have compounding stress from other stressful events in addition to stress from rearing a child with ASD. Siblings of children with ASD are at almost 20% higher risk of also being diagnosed with ASD.[27] With the prevalence of autism continually increasing and more children being diagnosed with autism, the need for research specific to these families increases. This study will examine pre-pregnancy and prenatal stressful life events in relation to ASD and non-typically developing (Non-TD) risk in a high familial risk cohort study.

With inconsistent findings on the association between stressful life events and risk of ASD, this study measured stress by using three methods and examined their respective associations with neurodevelopmental outcome in a high familial risk population.

Stressful Life Events (SLEs)

The preconception period, though less studied, is equally as important as the pregnancy period because it is a time when a mother's body can be affected in ways that could later have an effect on her pregnancy and subsequently, her child's development once born. To date, the authors do not know of any studies that have explored the association of preconception stressful life events (SLEs) and neurodevelopmental outcome in the child. However, studies have examined preconception stress in association with other pregnancy and child outcomes.[28,29] One investigation suggests that posttraumatic stress disorder (PTSD) experienced prior to conception may predict child negative affectivity when the child was 3-5 years of age.[28] Another study found that maternal stress experienced before conception was associated with shorter gestation.[29] Existing literature has also explored the relationship between SLEs in the prenatal period and neurodevelopmental outcome. Prenatal stress has been captured in various ways, including biomarkers for stress, namely cortisol, induced stress in animal models, and stressful life events the mother experiences during pregnancy. Previous investigations suggest a few biological pathways by which maternal stress during pregnancy could be associated with child neurodevelopment. There is correlational evidence showing that environmental factors experienced during pregnancy may interrupt the fetus's brain development when the central nervous system is still forming, and these environmental factors may be what causes atypical trajectory of neurodevelopment. Prenatal environmental exposures have been associated with risk of schizophrenia or other psychotic disorders.[30-32] In particular, there is abundant literature on prenatal stress suggesting that fetal programming is mediated by the effects of prenatal exposures on the fetus's developing hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis regulates the body's stress-response system[33] and is highly sensitive to experiences in early life, including the maternal stress during pregnancy as an environmental exposure to the fetus.[34]

In regards to ASD specifically, one Iranian case-control study concluded that stressful events during pregnancy, measured by failure to achieve life goals, having high debt, having conflict in the marriage or with the spouse's family, changes to sleeping habits, and sexual difficulties were higher in mothers that had children with ASD compared to mothers with typically developing children. Thus, the study concluded that these stressful events may be risk factors for developing ASD in the child,[35] though alternative explanations (e.g., maternal social conflicts or difficulties being due to autistic traits in the mother) cannot be ruled out. A retrospective study from 1990 of mothers who received prenatal care in a Pennsylvania clinic found an association not with a single life event but with experiencing family discord during pregnancy as a risk factor for ASD,[22] suggesting that associated risk could differ based on the types of events experienced.

Though these two studies found positive associations, most studies of prenatal stress through SLEs, such as death of a child, spouse, or first degree relative,[21,26] exposure to rocket attacks,[24] or days without electricity during an ice storm,[36] were not associated with risk of ASD. These studies looked at individual SLEs but did not consider the potential risk in experiencing multiple SLEs during pregnancy. To the authors' knowledge, this dissertation will be the first to examine whether there is a compounding effect of SLEs on the risk of neurodevelopmental outcomes, where pregnant women might have experienced more than one SLE during her pregnancy.

Perceived Stress

With inconsistent findings on the association between stressful life events and risk of ASD, the authors of this study hypothesize that it might not be the stressful events experienced themselves that are associated with an increased risk, but instead increased risk could relate to how a mother perceives these events. This hypothesis is based on theory and empirical evidence that the impact of stressors depends on how they are appraised and on the perceived coping resources available.[37] To the authors'

knowledge, this is the first prospective study to analyze perceived stress, which takes into account all types of stressors and how they could impact the mother.

Urinary Cortisol

Aside from measuring stress as SLEs or perceived stress, studies have also measured various biomarkers of stress, including oxidative stress markers like blood vitamin metabolite levels, glutathione peroxidase, and methionine.[38] Measuring stress biomarkers is important as it can help indicate changes to the body caused by specific stressors and help in creating interventions for management or reduction of stress, thus reducing risk of developing stress-related disorder.[39]

Cortisol is a glucocorticoid that plays an important role in the development of the fetal brain and other organs. The HPA axis is one of the body's primary stress-response systems, and fetal exposure to glucocorticoids affects programming of the HPA axis.[33] Corticotropin-releasing hormone (CRH) is synthesized from the hypothalamic paraventricular nucleus, which then stimulates the release of adrenocorticotropic hormone (ACTH). ACTH promotes the production of cortisol from the adrenal cortex and feeds back to modulate HPA activity.[40] In pregnancy, CRH is synthesized from the placenta and changes the regulation of the maternal HPA axis.[41,42] The mother's cortisol level is expected to increase at least two-fold throughout pregnancy.[43] During this time the fetus is exposed to increasing concentrations of cortisol.

One hypothesized pathway for maternal stress affecting the fetus's neurodevelopmental outcome is through the HPA axis. While the fetal hypothalamus begins forming around 9-10 weeks of gestation, it is not fully able to function until early in the second trimester of pregnancy.[44] It is theorized that ASD can develop because the fetus begins to experience the mother's stress during the second trimester. High stress could cause the fetus's HPA axis function to be altered to a higher set point or greater reactivity, which in turn could suppress the fetus's immune response. During the gestational period when the

blood-brain barrier (BBB) of the fetus is not fully developed, antibodies and other larger molecules have greater access to the brain.[45] Trauma or stress increases BBB permeability which enhances the risk of exposing the brain to environmental stimuli and insults that could impact neurodevelopment,[46] resulting in atypical development, such as ASD.[47]

Cortisol is the body's main stress hormone and is the most common way to measure stress response biologically. Measurement of cortisol prenatally has been widely assessed experimentally in rodent and non-human primate animal models and found to be associated with autism-like behaviors[7-10]. Prenatal cortisol has been less commonly studied experimentally in humans due to the ethical concerns of inducing prenatal stress and there have been few prospective studies to investigate the association of prenatal cortisol and neurodevelopmental outcomes.

The common methods for measuring cortisol have primarily been through salivary or serum samples. However, these sample collections are practically challenging to collect due to the nature of cortisol secretion being episodic and exhibiting a circadian rhythm.[48] Cortisol concentrations in the human body are at their highest in the morning and decline throughout the day. To accurately measure cortisol and compare across participants would require salivary or serum samples to be collected at the exact same time with repeat collections throughout the day. This approach was not feasible with this cohort due to staffing availability and burden on pregnant women and their families. Using 24-hr urine samples for cortisol is an established clinical collection method used for screening in Cushing's syndrome in which there is an overproduction of cortisol, called hypercortisolism.[49] Cortisol production rate is difficult to measure directly so the best method for diagnosis of hypercortisolism is through daily urine free cortisol excretion, measured in a 24-hr urine collection where creatinine is also determined to evaluate the completeness of the urine collection. There is an upper limit of normal daily urine free cortisol excretion.

Laboratories take into account pregnancy when determining 24-hr urine collection completeness, in which the upper limit of daily cortisol production rate and urine free cortisol excretion are elevated.

Because detecting cortisol production for Cushing's, a disease detecting overproduction of cortisol, is an accurate and trusted method, laboratories comfortably use this 24-hr urinary assay for measuring cortisol when the gold standard salivary cortisol is not feasible or available[48]. This study is, to the authors' knowledge, the first to use 24-hr urine samples during pregnancy to measure cortisol in association with child ASD and Non-TD neurodevelopmental outcomes.

Previous studies have found diurnal cortisol rhythms to differ based on racial and ethnic background, with Black and Hispanic groups having more subtle declines throughout the day compared to whites.[50,51] This study examined if race interacts with cortisol in the association between cortisol and neurodevelopmental outcome. The associations between prenatal cortisol and ASD were hypothesized to be stronger in non-white mother-child pairs than white mother-child pairs, due to cumulative effects of known cortisol pattern differences and other (unmeasured) stressors related to racism and discrimination. Additionally, due to the unequal ASD prevalence in males and females (4:1)[52] and biological plausibility of sex difference in ASD and Non-TD etiology,[53,54] this study also investigated if there is an interaction between sex and cortisol in the association between cortisol and neurodevelopmental outcome.

To date, the authors are aware of one study that has prospectively studied the association of fetal cortisol exposure and ASD symptoms at age 5.[33] The study found that fetuses exposed to lower levels of maternal cortisol were associated with more ASD symptoms in boys. The study used the Social Communication Questionnaire to gauge ASD symptoms. This present study prospectively investigated diagnosed ASD as the outcome, where expert clinicians assessed and confirmed the ASD diagnosis of the child.

Maternal Exposure in Relation to Race

Types of exposures, including stress, can differ across races and can be another result of racial disparities. A mother's socioeconomic status can play a role in her environment and what she is surrounded by. Moreover, since socioeconomic status is associated with race and there are many inequities faced by difference races, a mother's environment and her exposures can relate to her race.[55] These disparities that play a part in harmful exposures or lack of adequate health care and/or resources must be identified as early as possible in order to optimize long-term health of the child.

As a whole, the high and increasing prevalence in conjunction with the economic burden of ASD[56,57] present a pressing public health concern for society as a whole.[56] Specifically, the economic burden of ASD is heavily experienced by affected individuals and their families. These burdens are experienced differently by families of children with ASD when breaking down populations based on race or ethnicity, and existing literature shows striking differences that African American and Hispanic children have a higher prevalence of ASD compared to non-Hispanic white children,[58-60] yet the state of California is spending on average almost $2000 less per year for these families.[61] These non-white races and ethnicities experience systemic inequities when it comes to ASD services in the U.S. In fact, one ethnic group that continuously tends to be less studied, and thus overlooked for services, is the Asian American population.

There is sparse research looking into ASD in Asian American children, with existing studies showing conflicting results in prevalence compared to white children. Three studies in the US used surveillance data from the Autism and Developmental Disabilities Monitoring (ADDM) Network,[58] the California Department of Developmental Services (DDS),[59] and the San Francisco Bay Area[60] all found no statistically significant difference in ASD prevalence between Asian American and white children.[58-60]

One population-based study in Los Angeles County found prevalence of ASD in Asian Americans, specifically in Filipino and Vietnamese Asian American mothers compared to whites to be *higher*.[62] Differences in prevalence were explained by disparities in accessing and receiving diagnosis and treatment as well as language and cultural barriers in Asian American mothers. The authors suggested that dietary factors such as folic acid and Vitamin D deficiencies in Vietnamese and Filipino mothers might could explain the higher risk of ASD. Additionally, the authors suggested that foreign-born Filipino mothers have a large proportion employed in health care and may be at higher risk for infections that can affect fetal brain development.

Lastly, three studies show that ASD prevalence is *lower* in Asian Americans compared to whites.[63-65] The rationale for the lower Asian American ASD prevalence included more limited access to diagnostic services, availability of culturally competent diagnosticians, records that lack documentation of developmental concern, parental awareness, and speculation that minority children are under-screened for ASD by health professional.[64,65]

With limited literature examining Asian prevalence of ASD in the US and results of these studies being highly variable, it is unclear if MPS risk differs for Asian American families compared to white or other races. Without this knowledge, it is difficult to holistically assess diagnostic and intervention programs that specifically focus on this subpopulation. This study will be the first to examine prenatal perceived stress in relation to neurodevelopmental outcomes determined using a standardized protocol, stratified by race (non-Hispanic white, Asian, or Other). Results could uncover what could be true differences between non-Hispanic white and Asian American communities, but more plausibly could be differences due to other factors, such as cultural factors, stigma about mental health, and decreased access to resources.

The overarching objective of this study was to examine the association between prenatal maternal stress and neurodevelopmental outcome in the child. Subobjectives included examining this relationship stratified by race. In Chapter 1, the association between prenatal stressful life events and neurodevelopmental outcome was examined. In Chapter 2, the association of prenatal perceived stressful events and neurodevelopmental outcome was examined. In Chapter 3, the association of prenatal maternal cortisol and neurodevelopmental outcome was examined. The study population is a unique group of high familial risk mothers that have already had at least one diagnosed with ASD and so the subsequent child in MARBLES has enhanced risk of developing ASD.

Chapter 1

Maternal Preconception and Prenatal Stressful Life Events in Association with Child Neurodevelopmental Outcome in the MARBLES Study: A High Familial Risk Cohort

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ABSTRACT

Background and Objective: Existing literature findings on prenatal stress and its association with autism spectrum disorder (ASD) in the child are varying. While several types of prenatal stressful life events (SLEs) were not previously associated with higher likelihood of ASD, prenatal stress in the form of family discord was associated with increased likelihood of child ASD in one study. The effects of prenatal SLEs may be compounded among pregnant mothers who are already raising a child with ASD. This study was the first to examine preconception and prenatal SLEs in a high familial risk cohort in association with ASD diagnosis or other Non-typically developing (Non-TD) outcomes in the child, such as speech-language problems or learning difficulties.

Methods: The prospective longitudinal MARBLES cohort included 317 women with at least one child with ASD who then became pregnant with another child. Mothers were interviewed once during pregnancy and once at the end of their pregnancy to collect data on if they had experienced any of the following SLEs: 1. Changing or losing jobs, 2. Death of a close family member or close friends, 3. Divorce, separation from spouse/family member/someone close, serious difficulties or disagreements with relatives/neighbors/in-laws, 4. Legal problems, including immigration, 5. Financial problems, including foreclosure, 6. Moving or having a family member move into the household, and 7. Other major stressful events not already specified.

Child neurodevelopment was assessed longitudinally from birth through three years of age by trained psychologists who administered the Autism Diagnostic Observation Schedule (ADOS) and the Mullen Scales of Early Learning (MSEL). An algorithm based on ADOS and MSEL scores previously published by the Baby Siblings Research Consortium[2] was used to classify children with ASD, Non-TD, or typically developing (TD) outcomes.

Multinomial logistic regressions were fitted for the ASD and Non-TD outcome classifications with TD as the reference, controlling for maternal race/ethnicity and socioeconomic status for each of the seven SLEs separately, for a composite binary independent variable indicating whether 1+ SLE was experienced, and for how many SLEs a mother experienced during 6 months prior to conception through pregnancy.

Results: Experiencing legal problems, including immigration difficulties, was significantly associated with an increased risk of Non-TD outcome in the child (RRR 4.15 95% CI (1.29, 13.33)), though these results need to be interpreted with caution due to the small number of participants experiencing legal difficulties (n=6 in the Non-TD group). All other associations in the Non-TD group were non-significant. All associations with ASD were non-significant. Generally, SLEs during pregnancy were not associated with elevated risk of Non-TD or ASD in the child.

Conclusion: Findings agree with previous literature suggesting prenatal SLEs may not be risk factors for ASD and most were not strongly associated with Non-TD risk either. Experiencing legal problems, including immigration issues, was associated with increased risk of Non-TD and could be explained by rationale from previous investigations that found increased risk of neurodevelopmental disorders in children of non-US-born parents, with higher risk of ASD in those whose parents were from developing countries. These studies suggest that foreign-born parents might have been more frequently exposed to environmental pollutants, that they differ in socioeconomic status (SES), and that they differ from the local population in regards to pregnancy risk factors, like obesity or low SES. Additionally, non-US-born parents might face cultural and language barriers that could defer treatment of pregnancy complications. Future studies measuring stress using biomarkers or perceived stress are warranted to further understand the true association of prenatal stress and risk of ASD.

BACKGROUND

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by atypical development of social skills and language, and restricted or repetitive interests and behaviors.[1] In recent years, attention to ASD has increased due to the continual increase in prevalence. The CDC recently reported that 1 in 44 children in the United States was diagnosed with ASD in 2018, which is a nearly 23% increase since the reported estimate of 1 in 54 children just two years prior.[2] It is unclear whether this increase is due to increasing awareness and improved detection or due to a true increase in the prevalence of this condition, as the etiology of ASD is still unknown.

The prenatal period is a sensitive time when a mother's behaviors and exposures can affect her child's long-term health.[3] Like genetic factors, many prenatal environmental factors can have lasting effects on the neurodevelopment of the child.[4-6] Identifying prenatal exposures and understanding how they influence ASD risk is an area of research that is developing but warrants more inquiry. Equally important, and even less studied, is the preconception period when a mother's body can be affected in ways that may later have an effect on her pregnancy and subsequently, her child's development once born.

To date, the authors do not know of any studies that have explored the association of preconception stressful life events (SLEs) and neurodevelopmental outcome in the child. However, existing literature have examined preconception stress with other effects on the child.[28,29] One investigation suggests that posttraumatic stress disorder (PTSD) experienced prior to conception may predict child negative affectivity when the child was 3-5 years of age.[28] Another study found that maternal stress experienced before conception was associated with shorter gestation.[29] Existing literature has also explored the relationship of SLEs and neurodevelopmental outcome in the prenatal period. Prenatal stress has been captured in various ways, including biomarkers for stress, namely cortisol, induced stress in animal

models, and stressful life events the mother experiences during pregnancy. Previous investigations suggest a few biological pathways by which maternal stress during pregnancy could be associated with child neurodevelopment. There is correlational evidence showing that environmental factors experienced during pregnancy may interrupt the fetus's brain development when the central nervous system is still forming, and these environmental factors may be what causes atypical trajectory of neurodevelopment. This has been shown to be associated with child outcomes of schizophrenia or other psychotic disorders.[30-32] In particular, there is abundant literature on prenatal stress suggesting that fetal programming is mediated by the effects of prenatal exposures on the fetus's developing hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis regulates the body's stress-response system[33] and is highly sensitive to experiences in early life, including the maternal stress during pregnancy as an environmental exposure to the fetus.[34]

In regards to ASD specifically, one Iranian case-control study concluded that stressful events during pregnancy, measured by failure to achieve life goals, having high debt, having conflict in the marriage or with the spouse's family, changes to sleeping habits, and sexual difficulties were higher in mothers that had children with ASD compared to mothers with typically developing children. Thus, the study concluded that these stressful events may be risk factors for developing ASD in the child,[35] though alternative explanations cannot be ruled out (e.g., maternal social conflicts or difficulties being due to autistic traits in the mother). A retrospective study from 1990 of mothers who received prenatal care in a Pennsylvania clinic found an association not with a single life event but with experiencing family discord during pregnancy as a risk factor for ASD,[22] suggesting that associated risk may differ based on the type of event experienced.

Though these two studies found positive associations, most studies of prenatal stress through SLEs, such as death of a child, spouse, or first degree relative,[21,26] exposure to rocket attacks,[24] or days without

electricity during an ice storm,[36] were not associated with risk of ASD. These studies looked at individual SLEs but did not consider the potential risk in experiencing multiple SLEs during pregnancy. To the authors' knowledge, this dissertation will be the first to examine if there is a compounding effect of SLEs on the risk of neurodevelopmental outcomes, where pregnant women may have experienced more than one SLE during her pregnancy.

The studies exploring the association of stress in pregnant women and the risk of ASD are limited mainly to retrospective studies, and those that are prospective studies are limited in the type of stress event they measure. Additionally, maternal stress might be more of a concern for a child at high familial risk due to the mother already having a child diagnosed with ASD, meaning that she will have compounding stress from other stressful events in addition to stress from rearing a child with ASD. Siblings of children with ASD are at almost 20% higher risk of also being diagnosed with ASD.[27] With the prevalence of autism continually increasing and more children being diagnosed with autism, the need for research specific to these families increases. This study will examine pre-pregnancy and prenatal stressful life events in relation to ASD and non-typically developing (Non-TD) risk in a high familial risk cohort study.

OBJECTIVE / HYPOTHESIS

The objective of this study is to examine in a high familial risk cohort prenatal SLEs in association with ASD or Non-TD in the child. As existing literature suggests, it is not necessarily hypothesized that any one type of SLE will be associated with increased risk of Non-TD or ASD outcome, but rather it is hypothesized that the frequency of SLEs experienced will be the driver of higher risk;[66] the more SLEs a mother experiences during pregnancy, the higher the risk will be of Non-TD or ASD outcome in her child.

METHODS

Study Population

This study includes 317 women enrolled in the MARBLES study[67] who had at least one child with ASD and subsequently became pregnant with another child. These women are at elevated risk[27] for having another child who will develop ASD.

Eligibility and recruitment

Mothers eligible for MARBLES were identified through the California Department of Developmental Services (DDS), which periodically provide the study with updated lists of families that have at least one child who received services of autism. DDS provides this for all residents of California regardless of place of birth, religion, or financial resources. Families on these DDS lists are mailed a letter notifying them that the study will contact them to assess their interest and eligibility for participation, unless they opt out within two weeks by calling or emailing the study. Study personnel then call potential participants and determine eligibility with them. Participants must be: a) at least 18 years old, b) the biological parent of a child with ASD (or carrying the child of a male that is a biological father of a child with ASD), c) pregnant or planning a pregnancy, d) able to speak, read, and understand English and the child will be raised with English being a primary language, and e) residing in the study catchment area of a 2-hour drive radius from the Davis/Sacramento area.

Inclusion criteria consisted of confirmed diagnosis of ASD in at least one older full or half sibling of the child of interest in the study. Participants in this current study also had a final diagnosis at 36 months by the year 2017, meaning that they did not leave the study before they reached the final visit.

Child Neurodevelopmental Assessment

Child neurodevelopment was assessed longitudinally from birth through three years of age by trained clinicians at the UC Davis MIND Institute who administered the Autism Diagnostic Observation Schedule (ADOS) and the Mullen Scales of Early Learning (MSEL). The ADOS is a semi-structured interview during which the clinician observes social interaction, communication, play, and imaginative use of materials.[68] The MSEL measures cognitive functioning using subscales that measure fine motor, visual reception, and expressive and receptive language.[69]

An algorithm was then used to classify children as ASD, typically developing (TD), or non-typically developing (non-TD) based on their scores across the two assessments. Non-TD include those that had low MSEL scores and/or elevated ADOS scores. The algorithm used is a previously published method from the Baby Siblings Research Consortium.[70] Children with ASD outcomes (*n*= 75) had scores over the ADOS cutoff and met the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for ASD. Children with non-TD outcomes (*n*=44) had scores within three points of the ADOS cutoff and/or MSEL scores 1.5 to 2 standard deviants below average.[27] The remaining children were classified as TD (*n*=198). Child outcome at 36-months was used in this present study.

Exposure Assessment

Stressful Life Events

Mothers were asked at the end of their pregnancy if they had experienced any of 16 specified events listed in **Table 1** at any point 6 months prior to conception through pregnancy. There was also a concluding question to ask if they experienced any other major events not already specified, in which they could specify the event(s). Because some of the 16 events had low frequencies, the original events were then collapsed into 7 major events, shown below and in **Table 2**:

1. Changing or losing jobs

2. Death of a close family member or close friends

3. Divorce, separation from spouse/family member/someone close, serious difficulties or disagreements with relatives/neighbors/in-laws

4. Legal problems, including immigration

5. Financial problems, including foreclosure

6. Moving or having a family member move into the household

7. Other major stressful events not already specified; participant is asked to specify event(s)

SLE Quantification

In addition to analyzing the seven major events listed above, analyses were also performed to examine if exposure to one or more compounding stressful events 6 months prior to conception through pregnancy was a risk factor. To do this, regressions examined total events of 0, 1, 2, 3, and 4+ events experienced in the index time.

Confounding Assessment

A Directed Acyclic Graph (DAG) (**Figure 1**) was created based on existing literature to identity potential confounding factors *a priori*. All backdoor paths were identified and blocked with the minimally sufficient set of two potential confounders: maternal race/ethnicity and home ownership as a proxy for socioeconomic status (SES), which was not directly measured.

Race and ethnicity were collected from participants through multiple sources, which included the Environmental Exposure Questionnaire phone interview, the Family Information Form, the MARBLES database tracking system, and California birth files. Responses from four sources were self-reported by the participant. To determine race, participants were asked if they identified as: white, Black/African American, American Indian/Alaska Native, Asian, Native Hawaiian/Other Pacific Islander, Other, or more than one race. To determine ethnicity, participants were asked if they identified as Hispanic or not

Hispanic. Because this information was collected multiple times, if responses did not align, MARBLES programmers manually reviewed participants to determine any errors and correctly classified race/ethnicity.

Home ownership was collected through the Environmental Exposure Questionnaire phone interview, where participants self-reported if they owned a home or were a renter.

Covariate distributions were first checked graphically to confirm suitable variation was present. Each potential confounding covariate was examined with bivariate analysis for association with exposure and outcome separately. Both potential confounders, home ownership and maternal race/ethnicity, were found to be associated with outcome, but were not associated with exposure. However, they were still selected to continue in the next step of model building due to their selection from the DAG (**Figure 1**). Covariates were then individually added to the model. Covariates that, when added to the model individually, changed the beta value of the exposure of interest by at least 10% were selected for a full model. These covariates were then placed into step-up model building where the covariate that changed the effect estimate of the exposure of interest the most was added first. The final model included the exposure of interest, outcome, and the two covariates: maternal race and home ownership.

Statistical Analysis

Multinomial logistic regressions were fitted for ASD and Non-TD outcomes, controlling for maternal race/ethnicity and homeownership for each of the seven SLEs separately and for a composite binary independent variable indicating whether 1+ SLE was experienced. Regressions were also fit, controlling for the same factors above, for each of the compounding total numbers of SLEs experienced during pregnancy (1, 2, 3, and 4+, as a categorical variable, with 0 stressful life events being the reference for analysis). The Cochran-Armitage test was performed to examine if there was a dose-trend present for

compounding SLEs. Because child neurodevelopmental outcome was a 3-level outcome, the trend test was performed separately for ASD vs. TD and then for Non-TD vs. TD.

Because ASD is more prevalent in males than females (4:1 ratio)[52] and there is biologic plausibility for sex differences in ASD and Non-TD etiology,[54,71] stratification for child sex was examined in the association between dichotomous exposure to SLEs during pregnancy and child neurodevelopmental outcome.

All statistical analyses were performed with SAS version 9.4 (Institute Inc. Carny, NC, USA). *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Participants from the three outcome groups (TD, ASD, Non-TD) were similar on maternal race/ethnicity and homeownership status. Characteristics are presented in **Tables 3 and 4**. The majority of participants were non-Hispanic white (52.37%), followed by Hispanic (20.19%), Asian (16.09%), Mixed Race/Other (6.31%), and Black/African American (5.05%). The majority of participants were homeowners (58.25%). There were significant associations of maternal marital status, delivery payer, and child sex with child neurodevelopmental outcome. There was also a significant association in child year of birth with prenatal SLE exposure. The breakdown of characteristics by neurodevelopmental outcome group and by exposure can be found in **Tables 3 and 4**.

Relative risk ratios (RRR) and confidence intervals are presented in **Table 5**, with a graphical representation of the log of relative risk ratios in **Figure 2**. Experiencing legal problems, including immigration difficulties, was significantly associated with an increased risk of Non-TD outcome in the child (RRR 4.15, 95% CI (1.29, 13.33)), though these results need to be interpreted with caution due to the small number of participants experiencing legal difficulties (n=6 in the Non-TD group). All other SLEs

were not found to be statistically significant, although experiencing financial problems, including foreclosure difficulties, was borderline significant for increased risk of Non-TD outcome in the child (RRR 2.23, 95% CI (0.99, 4.98)). A dichotomous exposure of experiencing any SLE during pregnancy was non-significant. The compounding number of SLEs experienced during pregnancy (0, 1, 2, 3, 4+) was also non-significant (**Table 6**), nor was there a dose-trend present (ASD vs. TD *p*-value 0.98; Non-TD vs. TD *p*-value 0.16).

When stratified by child sex, relative risk ratios in females compared to males for whether or not mothers experienced and SLE during pregnancy were in the same direction for the Non-TD group but in different directions in the ASD group, though not meaningfully different across groups (**Table 7**).

Generally, SLEs experienced 6 months prior to conception through pregnancy were not associated with elevated risk of Non-TD or ASD outcome in the child.

DISCUSSION

Findings agree with previous literature suggesting prenatal SLEs may not be risk factors for ASD and most were also not strongly associated with Non-TD risk. The increased risk of legal problems on Non-TD merits further exploration, though precision was low. Previous investigations have shown that risk of neurodevelopmental disabilities, namely intellectual disability, attention deficit hyperactivity disorder, and ASD, increases in children of immigrant and refugee parents.[72-74] Risk was associated with region of birth,[74] with higher risk associated with mothers born in developing countries.[73] Several explanations for the association of immigration and neurodevelopment have been suggested. One theory is that immigrant mothers have been more frequently exposed to environmental pollutants, like heavy metals.[72,75] Another justification states that immigrants differ from the population they emigrated to on factors such as obesity, low socioeconomic status, and risk factors associated with low SES, such as poor nutrition.[47,76-78] Another theory explains that immigrant mothers face cultural and language barriers

when seeking prenatal care,[79] which could defer their treatment when pregnancy complications are present, which are known risk factors for neurodevelopmental disorders.[80,81] Lastly, explanations considering psychosocial stress experienced during the premigration, migration, or post-migration periods may adversely affect fetal neurodevelopment through epigenetic mechanisms.[82]

The overall null findings may be explained by the fact that this study population was a high familial risk cohort. With mothers already having at least one child diagnosed with ASD before this MARBLES pregnancy, they have already had experience navigating all that this entails, which includes (but is not limited to) locating services for a diagnosis for her child, receiving and understanding the diagnosis, finding services for her child, learning new ways to interact with her child, etc. These mothers could be more resilient at handling stressful life events since their baseline stress levels might be higher compared to mothers that do not already have a child diagnosed with autism. Alternatively, it may not necessarily be the stressful events experienced themselves, but how the event is perceived by the mother that is associated with risk. Future studies measuring stress using biomarkers or perceived stress are warranted to further understand the true association of prenatal stress and risk of ASD.

Interestingly, some mothers reported some "other stressful events" they experienced during pregnancy or 6 months prior to conception as situations related to their older child being diagnosed with ASD. There were not enough mothers who reported this to analyze this as a separate stressful life event. Similarly, a number of mothers also reported pregnancy difficulties or miscarriages as another stressful life event. Both of these warrant further exploration to test whether they may affect risk of autism in the current pregnancy. In the present study, results were non-significant but relative risk ratios for ASD were well below 1.0, suggesting that stressful life events are in the protective direction. This might be explained by selection (and survival) bias in which experiencing prenatal SLEs leads to fetal loss in those fetuses that would have grown to develop ASD, especially in highly genetically susceptible families that

are at elevated risk of developing ASD. The fetuses that did survive will have been less influenced by the stressful events than those fetuses that did not survive to be selected into the study population. Thus, the true association cannot be studied because of this survival bias.

This study had a number of limitations, which include small total sample size and small sample sizes of non-white races. Because these data were analyzed from pre-existing questionnaires, we were not able to tease apart the preconception and prenatal periods to analyze the two periods separately. SLEs were also not able to be analyzed by trimester. Analysis by trimester could be important since previous studies have shown associations with stress (specifically by timing in the second and third trimesters) with ASD.[22,23] Strengths include the novel research performed in a high familial risk population. Clinical assessments determining child neurodevelopmental outcome were performed using gold standard methods administered by expert trained clinicians at the UC Davis MIND Institute. Additionally, this was a prospective cohort so mothers did not have to recall a long period of time when completing phone interviews and questionnaires, thus reducing recall bias. Exposure information was collected before outcome was assigned so there is no question of the temporal relationship between exposure and outcome in this cohort study.

Overall, results of this high familial risk study confirm findings in the general population. Experiencing SLEs in preconception and pregnancy do not appear to be associated with increased risk of non-typical development or ASD. Future studies should investigate different measures of stress, namely perceived stress or cortisol levels, in order to more thoroughly understand the associations between prenatal stress and risk of neurodevelopmental outcomes.

APPENDIX

Table 1. List of stressful events asked in questionnaire

Stressful Life Events Asked in Questionnaire	
1. Changing jobs	10. Move to a new house or apartment
2. Losing job or remaining unemployed for one month or longer	11. Addiction or mental problem
3. Serious illness or injury	12. Separated from family or a close friend
4. Death of a close family member	13. Serious problems or disagreements with relatives, neighbors, or in-laws
5. Separation, divorce, or serious difficulty with someone close to you	14. Experience any natural or man-made disasters
6. Serious problems related to immigration	15. Victim of violence or crime
7. Serious legal problems not related to immigration	16. Family member moved into household
8. Serious problems related to foreclosure	17. Other major events, not already specified; participant asked to specify event(s)
9. Serious financial problems not related to foreclosure	

Table 1. Mothers were asked if they experienced any of the 17 stressful life events listed during the index time period of 6 months prior to conception through the end of pregnancy.

Table 2. List of stressful events – collapsed for analysis

Stressful Life Events Collapsed for Analysis
1. Changing or losing jobs
2. Death of a close family member or close friends
3. Divorce, separation from spouse/family member/someone close, serious difficulties or disagreements with relatives/neighbors/in-laws
4. Legal problems, including immigration
5. Financial problems, including foreclosure
6. Moving or having a family member move into the household
7. Other major stressful events not already specified; participant asked to specify event(s)

Table 2. The 17 stressful life events listed in Table 1 were collapsed into these 7 categories for analyses due to low frequencies.

Table 3. Demographic characteristics of study participants, stratified by child neurodevelopmental outcome.

Characteristics	Child 36-month Neurodevelopmental Outcome								
	Row total		TD		ASD		Non-TD		*p*-value
	n	%	n	%	n	%	n	%	
Mother Race/Ethnicity									0.35
Non-Hispanic White	166	52.37	108	54.55	36	48	22	50	
Hispanic	64	20.19	39	19.7	15	20	10	22.73	
Black/African American	16	5.05	5	2.53	7	9.33	4	9.09	
Asian	51	16.09	33	16.67	11	14.67	7	15.91	
Mixed Race/Other	20	6.31	13	6.57	6	8	1	2.27	
Homeowner									0.07
No	129	41.75	73	37.24	37	52.86	19	44.19	
Yes	180	58.25	123	62.76	33	47.14	24	55.81	
Maternal Education Level									0.21
Less than High School	9	2.84	4	2.02	3	4	2	4.55	
High school diploma/ GED	16	5.05	8	4.04	4	5.33	4	9.09	
Some college	129	40.69	72	36.36	37	49.33	20	45.45	
Bachelor's degree	102	32.18	74	37.37	19	25.33	9	20.45	
Graduate or Professional Degree	61	19.24	40	20.2	12	3.79	9	2.84	
Maternal Marital Status									0.001
Married or Living as Married	285	91.35	184	93.4	68	94.44	33	76.74	
Other (divorced, separated, single, widowed)	27	8.65	13	6.6	4	5.56	10	23.26	
Parents born inside or outside of the U.S.									0.34
Both parents born inside U.S.	197	65.67	132	68.04	35	55.56	30	69.77	
One parent born inside U.S.	52	17.33	30	15.46	16	25.4	6	13.95	

	Both parents born outside U.S.	51	17	32	16.49	12	19.05	7	16.28	
Delivery Payer										0.003
	Private	245	78.53	164	84.54	53	70.67	28	65.12	
	Public	67	21.47	30	15.46	22	29.33	15	34.88	
Child Year of Birth										0.1
	2006	1	0.32	1	0.51	0	0	0	0	
	2007	14	4.42	11	5.56	1	1.33	2	4.55	
	2008	36	11.36	29	14.65	3	4	4	9.09	
	2009	46	14.51	29	14.65	10	13.33	7	15.91	
	2010	35	11.04	23	11.62	9	12	3	6.82	
	2011	19	5.99	12	6.06	6	8	1	2.27	
	2012	18	5.68	9	4.55	8	10.67	1	2.27	
	2013	34	10.73	13	6.57	10	13.33	11	25	
	2014	37	11.67	21	10.61	11	14.67	5	11.36	
	2015	38	11.99	27	13.64	6	8	5	11.36	
	2016	38	11.99	22	11.11	11	14.67	5	11.36	
	2017	1	0.32	1	0.51	0	0	0	0	
Child Sex										0.01
	Female	136	42.9	96	48.48	21	28	19	43.18	
	Male	181	57.1	102	51.52	54	72	25	56.82	

	Total			**TD**			**ASD**			**Non-TD**			***p*-value**
	n	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	
Maternal Age at Delivery	317	34.55	4.9	198	34.8	4.9	75	34.49	4.97	44	33.55	4.44	0.31
Paternal Age at Delivery	312	36.75	5.5	196	36.6	5.6	73	37.59	5.4	43	39.91	5.41	0.25

Table 3. Demographics of study participants, stratified by neurodevelopmental outcomes, are shown. TD = Typical Development; ASD = autism spectrum disorder; Non-TD = Non-Typical Development. P-values were obtained from chi-square tests.

Table 4. Demographic characteristics of study participants, stratified by prenatal SLE exposure.

Characteristics		Prenatal SLEs Exposure						*p*-value
		Row total		No SLEs during pregnancy		1+ SLEs during pregnancy		
		n	%	n	%	n	%	
Mother Race/Ethnicity								0.09
	Non-Hispanic White	141	51.27	35	58.33	106	49.3	
	Hispanic	57	20.73	8	13.33	49	22.79	
	Black/African American	15	5.45	0	0	15	6.98	
	Asian	46	16.73	13	21.67	33	15.35	
	Mixed Race/Other	16	5.82	4	6.67	12	5.58	
Homeowner								0.18
	No	118	43.7	20	35.71	98	45.79	
	Yes	152	56.3	36	64.29	116	54.21	
Maternal Education Level								0.33
	Less than High School	8	2.91	3	5	5	2.33	
	High school diploma/ GED	14	5.09	2	3.33	12	5.58	
	Some college	114	41.45	21	35	93	43.26	
	Bachelor's degree	82	29.82	17	28.33	65	30.23	
	Graduate or Professional Degree	57	20.73	17	28.33	40	18.6	
Maternal Marital Status								0.81
	Married or Living as Married	247	90.48	52	89.66	6	10.34	
	Other (divorced, separated, single, widowed)	26	9.52	6	10.34	20	9.3	
Parents born inside or outside of the U.S.								0.74
	Both parents born inside U.S.	165	62.98	33	58.93	132	64.08	

	One parent born inside U.S.	49	18.7		11	19.64		38	18.45	
	Both parents born outside U.S.	48	18.32		12	21.43		36	17.48	
Delivery Payer										0.12
	Private	211	77.57		51	85		160	75.47	
	Public	61	22.43		9	15		52	24.53	
Child Year of Birth										0.004
	2006	1	0.36		1	1.67		0	0	
	2007	4	1.45		4	6.67		0	0	
	2008	10	3.64		5	8.33		5	2.33	
	2009	45	16.36		10	16.67		35	16.28	
	2010	33	12		6	10		27	12.56	
	2011	19	6.91		5	8.33		14	6.51	
	2012	18	6.55		5	8.33		13	6.05	
	2013	33	12		5	8.33		28	13.02	
	2014	37	13.45		8	13.33		29	13.49	
	2015	37	13.45		7	11.67		30	13.95	
	2016	37	13.45		4	6.67		33	15.35	
	2017	1	0.36		0	0		1	0.47	
Child Sex										0.89
	Female	117	42.55		26	43.33		91	42.33	
	Male	158	57.45		34	56.67		124	57.67	
		Total			**No SLEs during pregnancy**			**1+ SLEs during pregnancy**		***p*-value**
		n	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**
Maternal Age at Delivery		317	34.55	4.88	60	34.45	4.31	215	34.51	4.84	0.93
Paternal Age at Delivery		312	36.75	5.53	60	36.96	5.27	211	36.66	5.67	0.72

Table 4. Demographics of study participants, stratified by prenatal SLE exposure, are shown. TD = Typical Development; ASD = autism spectrum disorder; Non-TD = Non-Typical Development. P-values were obtained from chi-square tests.

Table 5. Associations Between Prenatal Stressful Life Events and ASD or Other Non-Typical Development (Non-TD)

Stressful Life Event (n=*exposed/total)*		**Exposed**		**Relative Risk Ratios (vs. TD)**	**95% CI**
		n	**%**		
1. Experienced any stressful life event during pregnancy (yes/no) (n=215/275)	TD	130	78.31	REF	--
	ASD	53	75.71	0.8	(0.40, 1.62)
	Non-TD	32	82.05	1.27	(0.48, 3.34)
2. Changing or losing jobs (n=42/270)	TD	25	15.15	REF	--
	ASD	11	16.18	0.92	(0.40, 2.07)
	Non-TD	6	16.22	1.01	(0.37, 2.77)
3. Death of a close family member or close friend (n=58/271)	TD	40	24.24	REF	--
	ASD	11	16.18	0.61	(0.28, 1.29)
	Non-TD	7	18.42	0.71	(0.29, 1.76)
4. Divorce, separation from spouse/family member/someone close, serious difficulties or disagreements with relatives/neighbors/in-laws (n=110/270)	TD	61	37.2	REF	--
	ASD	32	47.06	1.47	(0.81, 2.68)
	Non-TD	17	44.74	1.28	(0.62, 2.68)
5. Legal problems, including immigration (n=18/267)	TD	8	4.97	REF	--
	ASD	4	5.8	1.22	(0.34, 4.33)
	Non-TD	6	16.22	4.15	(1.29, 13.33)
6. Financial problems, including foreclosure (n=68/268)	TD	37	22.84	REF	--
	ASD	15	23.19	0.96	(0.47, 1.95)
	Non-TD	22	40.54	2.23	(0.99, 4.98)
7. Moving or having a family member move into the household (n=76/270)	TD	47	28.66	REF	--
	ASD	15	22.06	0.62	(0.31, 1.26)
	Non-TD	14	36.84	1.29	(0.59, 2.83)
8. Other major stressful events not already specified (n=19/272)	TD	11	6.67	REF	--
	ASD	7	10.14	1.57	(0.54, 4.57)
	Non-TD	1	2.63	0.41	(0.05, 3.34)

Table 5. Results of the multinomial logistic regressions examining the relationship between the dichotomous stressful life events variable in the index time period and neurodevelopmental outcome as well as all of the seven stressful life events examined independently with neurodevelopmental outcome. Relative risk ratios and 95% confidence intervals are presented. A graphical representation of the results are presented in Figure 2.

Table 6. Associations Between Compounding SLEs Experienced in Pregnancy and ASD or Other Non-Typical Development

Quantity of Stressful Life Events (SLEs) Experienced During Pregnancy (n=total)	**TD** (REF)		**ASD** vs. TD				**Non-TD** (vs. TD)			
	Exposed		**Exposed**		**Relative Risk Ratio**	**95% CI**	**Exposed**		**Relative Risk Ratio**	**95% CI**
	n	%	n	%			n	%		
Experienced 0 SLEs (n=60)	36	21.69	17	24.29	REF	--	7	17.95	REF	--
Experienced 1 SLE (n=109)	67	40.36	27	38.57	0.8	(0.37, 1.74)	15	38.46	1.21	(0.43, 3.45)
Experienced 2 SLEs (n=60)	38	22.89	15	21.43	0.83	(0.35, 2.00)	7	17.95	0.94	(0.28, 3.12)
Experienced 3 SLEs (n=28)	17	10.24	6	8.57	0.61	(0.19, 1.94)	5	12.82	1.45	(0.37, 5.59)
Experienced 4+ SLEs (n=18)	8	44.44	5	27.78	1.07	(0.29, 4.02)	5	27.78	3.17	(0.73, 13.75)

Table 6. Results of the multinomial logistic regression examining the relationship between the sum of stressful life events experienced in the index time period and neurodevelopmental outcome. Relative risk ratios and 95% confidence intervals are presented. Because somewhat of a dose-trend was observed, the Cochran-Armitage test was performed separately for ASD vs. TD and then for Non-TD vs. TD but no trends were found (p-values 0.98 and 0.16, respectively).

Table 7. Associations Between Dichotomous Exposure to SLEs in Pregnancy and ASD or Other Non-Typical Development, Stratified by Child Sex

Females: Stressful Life Event (n=*exposed/total)*		**Exposed**		**Relative Risk Ratios (vs. TD)**	**95% CI**
		n	**%**		
Experienced any stressful life event during pregnancy (yes/no) (n=91/117)	TD	60	76.92	REF	--
	ASD	17	80.95	1.11	(0.28, 4.52)
	Non-TD	14	77.78	1.29	(0.32, 5.19)
Males: Stressful Life Event (n=*exposed/total)*		**Exposed**		**Relative Risk Ratios (vs. TD)**	**95% CI**
		n	**%**		
Experienced any stressful life event during pregnancy (yes/no) (n=124/158)	TD	70	79.55	REF	--
	ASD	36	73.47	0.69	(0.29, 1.63)
	Non-TD	18	85.71	1.19	(0.31, 4.68)

Table 7. Results of the multinomial logistic regression examining the relationship between dichotomous exposure to SLEs in pregnancy and neurodevelopmental outcome, stratified by child sex. Relative risk ratios and 95% confidence intervals are presented.

Figure 1. Directed Acyclic Graph (DAG) for Selection of Confounders

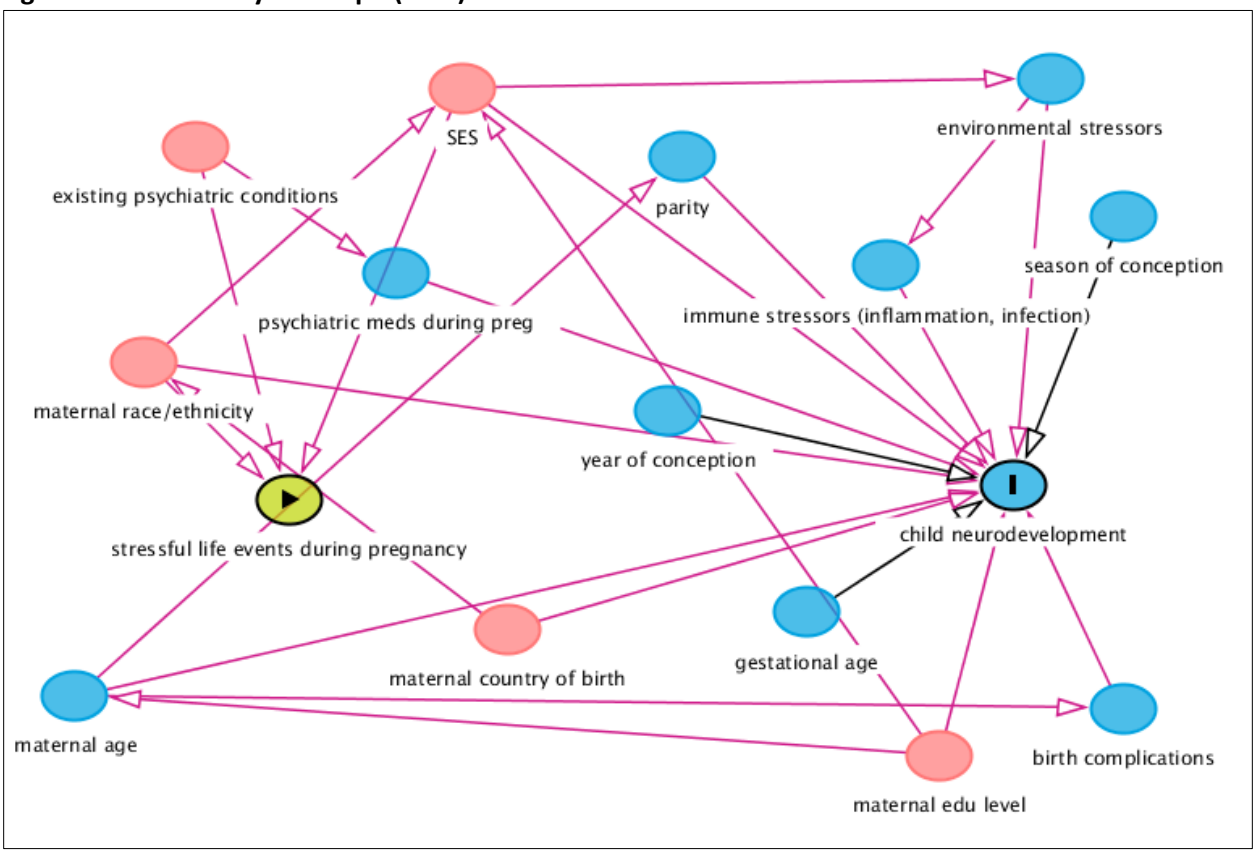

Figure 1. Directed Acyclic Graph created to select covariates to control for a priori. Exposure variable is stressful life events during pregnancy. Outcome is child neurodevelopment. Variables in blue are on the pathway from exposure to outcome. Variables in red are confounders. Variables selected as the minimally sufficient set to control for in order to block all backdoor paths were: existing psychiatric conditions, maternal race/ethnicity and home ownership as a proxy for socioeconomic status (SES). Data on existing psychiatric conditions were not available at the time of analysis so this covariate was omitted from the regression models.

Figure 2. Graphical representation of results showing Log(relative risk ratio) and 95% confidence intervals

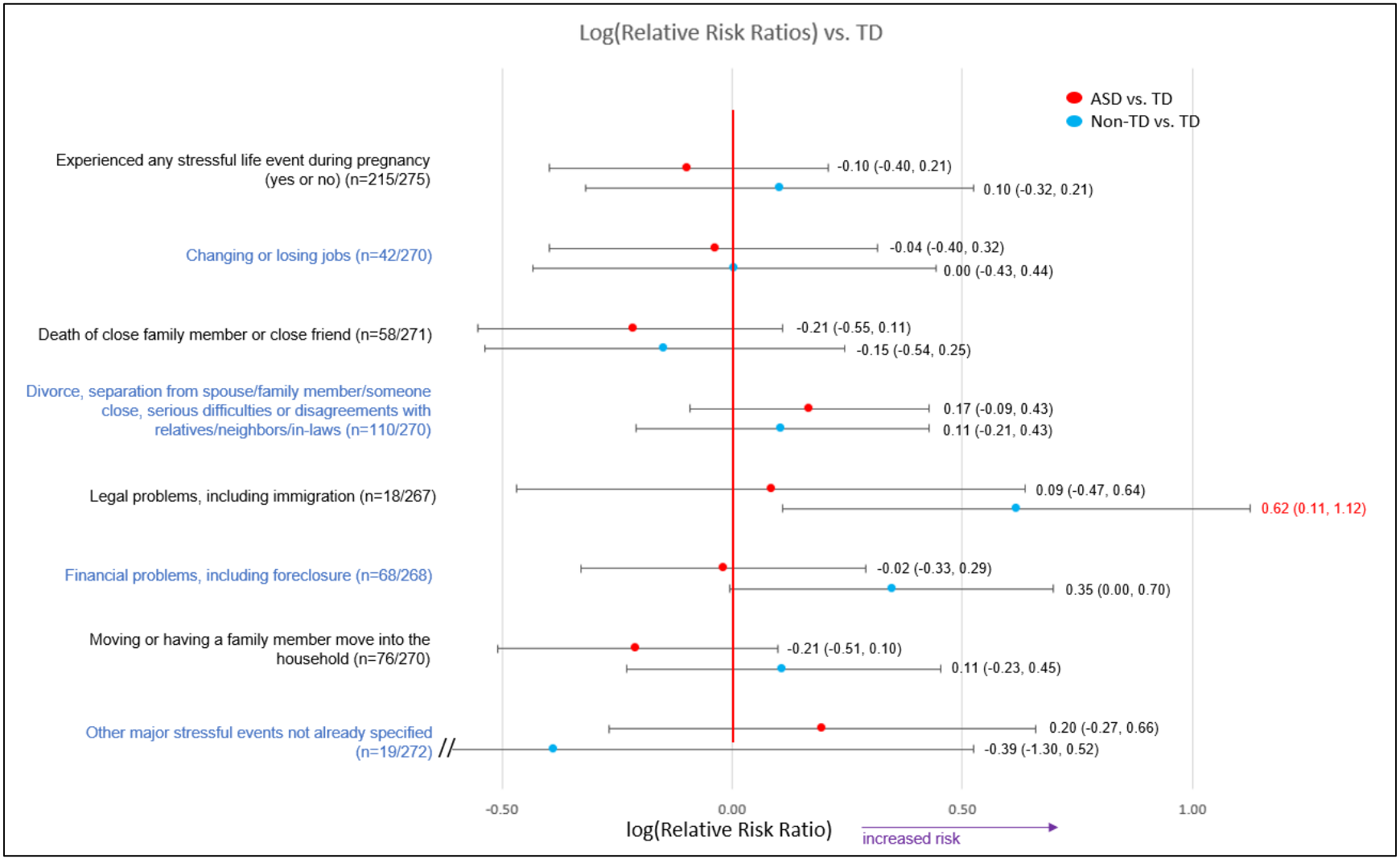

Figure 2. Log of relative risk ratios plotted with 95% confidence intervals for the dichotomous stressful life events exposure variable and the seven stressful life events separately. The association between experiencing legal problems, including immigration, in the Non-TD group was significant when compared to the TD group. All other association were non-significant for the Non-TD group. All associations were non-significant for the ASD group.

Chapter 2

Prenatal perceived stress as a risk factor for ASD and non-typical developmental outcome in MARBLES: A high familial risk cohort

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ABSTRACT

Background and Objective: The majority of previous investigations have found that experiencing stressful life events during pregnancy is not associated with neurodevelopmental outcomes. Because mothers may experience stressful life events differently, it is hypothesized that it may not be the stressful events experienced themselves that pose a risk to the child, but that the risk lies in how the mother perceives these life events. This study is the first to examine prenatal perceived stress in association with ASD and other non-typical development in the child.

Methods: This study includes 261 women from the prospective longitudinal Markers of Autism Risk in Babies: Learning Early Signs (MARBLES) cohort. Given that by design all of these mothers have had at least one other child with ASD, their current pregnancy is at high familial risk of developing ASD as well. Mothers completed the Perceived Stress Scale (PSS) Questionnaire at every prenatal MARBLES visit, which occurred twice a trimester. Child neurodevelopment was assessed longitudinally from birth through three years of age by trained psychologists at the UC Davis MIND Institute who administered the Autism Diagnostic Observation Schedule (ADOS) and Mullen Scales of Early Learning (MSEL). The Baby Siblings Research Consortium (BSRC) algorithm based on ADOS and MSEL scores was used to classify children with ASD, Non-TD, or typically developing (TD) outcomes at three years of age were used for the present study. Multinomial logistic regressions for this outcome (with TD as the reference group) were fitted with PSS average scores for each trimester as the exposure of interest and controlling for maternal race/ethnicity, maternal age, and home ownership. Additional multinomial logistic regressions were fitted to test the associations of PSS deviation scores in each trimester with the outcomes, controlling for the same covariates above. Lastly, multinomial logistic regressions examined the adjusted associations of PSS average trimester scores with outcome classifications, stratified by maternal race/ethnicity (non-Hispanic white, Asian, and Other). A sensitivity analysis was conducted to compare to the primary analysis and included additional potential confounders: maternal country of

birth, immune stressors during pregnancy, parity, ambient carbon monoxide, nitrogen oxide, nitrogen dioxide, ozone, adjusted PM10, and PM2.5.

Results: In trimester 1, a change in one point on the PSS scale was associated with a 10% increased relative risk for Non-TD (RRR 1.10, 95% CI (1.00, 1.21)) but no significant association was found for ASD. In trimester 2, a one point increase in PSS scale was associated with 8% relative risk for ASD (RRR 1.08, 95% CI (1.02, 1.14))). In trimester 3, a one point increase in PSS scale was associated with 8% increased relative risk for ASD (RRR 1.08, 95% CI (1.03, 1.14)). Associations were non-significant for Non-TD in trimesters 2 and 3. No significant differences in perceived stress and ASD or Non-TD outcome were found when stratified by child sex. Significant differences were also not detected when stratifying associations by maternal race/ethnicity.

Conclusions: Findings support the hypothesis that perceived stress is associated with increased risks of ASD and possibly Non-TD, relative to TD. This study suggests the possibility that stress reduction interventions during pregnancy could serve as preventative measures that help optimize the child's long-term health, though readers should be cautioned that there is potential that this is a non-causal association between perceived stress and neurodevelopmental outcome. Larger studies are needed to replicate these findings. Future studies should also examine biomarkers for stress in association with neurodevelopmental outcomes.

BACKGROUND

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by atypical development in three domains: social difficulties, rigid personality or behavior, and language delays or atypical development.[70] Within recent years, attention to ASD has increased due to the continual increase in prevalence. The CDC currently estimates that 1 in 44 children in the U.S. has ASD.[2] This rise is only partially explained by diagnosis at an earlier age, changes to diagnostic criteria, and inclusion of milder cases.[83] Researchers currently believe that genetic susceptibility and environmental factors both play a part in explaining the etiology of ASD, and research is ongoing to uncover the true etiology.[4-6,84,85]

Maternal prenatal stress (MPS) is a complex exposure that depends on many factors, including the mother's financial standing, family events and complications, and ability to provide her family with basic needs. Associations between maternal MPS and neurodevelopmental disorders in her offspring have been reported in previous studies.[7-20] Experimental animal models, using rodents and non-human primates, have found that induced MPS was associated with behavioral differences in the offspring, such as altered cardiovascular functioning, low birth weight, and delayed motor development.[7-10] However, difficulties of comparing rodent and non-human primate lifecycles to the human lifecycle make it hard to interpret these animal findings in the context of pregnant women. In some human epidemiologic studies, research indicates prenatal exposure to stress as being associated with risk of neurodevelopmental disorders[11-15] and has been found to be associated with specific neurodevelopmental disorders, including Attention Deficit Hyperactivity Disorder and ASD.[16-20]

However, human epidemiologic studies examining prenatal maternal stress and its association with ASD have varying findings. Three studies found increased risk of ASD in mothers exposed to MPS, measured by death of a first-degree relative,[21] family discord,[22] and broad recall of any stressful events.[17,23] However, a few studies found no association with ASD when measuring MPS through: exposure to

specific rocket attacks,[24] prospective collection of routine and major stressors,[25] or experiencing death of a close relative.[26] The discordant results might be explained by differing study designs. For example, the study examining death of a first-degree relative[21] was retrospective, while the study examining death of a close relative[26] was prospective.

With inconsistent findings on the association between stressful life events and risk of ASD, the authors of this study hypothesize that it may not be the stressful events experienced themselves that are associated with an increased risk, but instead increased risk could relate to how a mother perceives these events. This hypothesis is based on theory and empirical evidence that the impact of stressors depends on how they are appraised and on the perceived coping resources available.[37] To the authors' knowledge, this is the first prospective study to analyze perceived stress, which takes into account all types of stressors and how they could impact the mother.

This study will also be the first to examine prenatal perceived stress and neurodevelopmental outcomes in a high familial risk cohort study, in which pregnant mothers have already had at least one other child with ASD. Siblings of children with ASD are at about 10 times higher risk of also being diagnosed with ASD.[27] With the prevalence of autism continually increasing and more children being diagnosed with autism, the need for research specific to these families increases. Thus, this study will help fill the gap of knowledge about high-risk populations where research is still sparse. With these women already being at elevated risk of having another child with ASD, having a greater understanding of potential impacts of added stress during pregnancy could help inform prevention strategies for future families.

Types of exposures, including stress, can differ across races and can be another result of racial disparities. A mother's socioeconomic status can play a role in her environment and what she is surrounded by. Moreover, since socioeconomic status is associated with race and there are many inequities faced by difference races, a mother's environment and her exposures can relate to her race.[55]

These disparities that play a part in harmful exposures or lack of adequate health care and/or resources must be identified as early as possible in order to optimize long-term health of the child.

As a whole, the high and increasing prevalence in conjunction with the economic burden of ASD[56,57] present a pressing public health concern for society as a whole.[56] Specifically, the economic burden of ASD is heavily experienced by affected individuals and their families. These burdens are experienced differently by families of children with ASD when breaking down populations based on race or ethnicity, and existing literature shows striking differences that African American and Hispanic children have a higher prevalence of ASD compared to non-Hispanic white children,[58-60] yet the state of California is spending on average almost $2000 less per year for these families.[61] These non-white races and ethnicities experience systemic inequities when it comes to ASD services in the U.S.. In fact, one ethnic group that continuously tends to be less studied, and thus overlooked for services, is the Asian American population.

There is sparse research looking into ASD in Asian American children, with existing studies showing conflicting results in prevalence compared to white children. Three studies in the US used surveillance data from the Autism and Developmental Disabilities Monitoring (ADDM) Network,[58] data from the California Department of Developmental Services (DDS),[59] and the San Francisco Bay Area[60] all found no statistically significant difference in ASD prevalence between Asian American and white children.[58-60]

One population-based study in Los Angeles County found prevalence of ASD in Asian Americans, specifically in Filipino and Vietnamese Asian American mothers compared to whites to be *higher*.[62] Differences in prevalence were explained by disparities in accessing and receiving diagnosis and treatment as well as language and cultural barriers in Asian American mothers. The authors suggested that before immigrating to the U.S., Vietnamese mothers might have experienced stressful life events

from escaping wars and disasters, while foreign-born Filipino mothers have a large proportion employed in health care and may be at higher risk for infections that can affect fetal brain development.

Lastly, three studies show that ASD prevalence is *lower* in Asian Americans compared to whites.[63-65] The rationale for the lower Asian American ASD prevalence included access to diagnostic services, availability of culturally competent diagnosticians, records that lack documentation of developmental concern, parental awareness, and speculation that minority children are under-screened for ASD by health professionals.[64,65]

With limited literature examining Asian prevalence of ASD in the US and results of these studies being highly variable, it is unclear if MPS risk differs for Asian American families compared to white or other races. Without this knowledge, it is difficult to holistically assess diagnostic and intervention programs that specifically focus on this subpopulation. This study will be the first to examine prenatal perceived stress in relation to neurodevelopmental outcomes determined using a standardized protocol, stratified by race. Results could uncover what could be true differences between non-Hispanic white and Asian American communities, but more plausibly could be differences due to other factors, such as cultural factors, stigma about mental health, and decreased access to resources.

OBJECTIVE/HYPOTHESIS

The main objective of this study is to examine the association between prenatal perceived stress and child neurodevelopmental outcomes. A subobjective is to examine this association stratified by race. It is hypothesized that prenatal perceived stress will be significantly associated with ASD and Non-TD outcomes and that this association differs by race, with the association being stronger in non-white races. This could be because non-white mothers' perceived stress is compounded by structural racism, disparities in health care, microaggressions, stressors of assimilating, and lack of access to culturally sensitive support.

METHODS

Study population

Participants were from the Markers of Autism Risk in Babies: Learning Early Signs (MARBLES) study.[67] MARBLES is a prospective longitudinal cohort study that recruits women who are pregnant or planning a pregnancy and have at least one other child with autism. These women are at high familial risk of having their subsequent children diagnosed with ASD.[27] Eligible mothers are identified through the California Department of Developmental Services (DDS), which periodically provides the study with updated lists of families that have at least one child who received services of autism. DDS provides services for all residents of California regardless of place of birth, religion, or financial resources. Families on these DDS lists are mailed a letter notifying them that the study will contact them to assess their interest and eligibility for participation, unless they opt out within two weeks by calling or emailing the study. Study personnel then call potential participants and go through eligibility with them. Participants had to be: a) at least 18 years old, b) the biological parent of a child with ASD (or carrying the child of a male that is a biological father of a child with ASD), c) pregnant or planning a pregnancy, d) able to speak, read, and understand English and the child will be raised with English being a primary language, and e) residing in the study catchment area of living within a 2-hour radius from the Davis/Sacramento area.

For this present study, 261 mothers were selected if (1) they had provided at least one response to the Perceived Stress Scale questionnaire during pregnancy and (2) their child completed the study assessments at 3 years of age.

Child neurodevelopmental assessment

Child neurodevelopment was assessed longitudinally from birth through three years by UC Davis MIND Institute licensed clinical psychologists who administered the gold standard Autism Diagnostic Observation Schedule (ADOS)[68]. The Mullen Scales of Early Learning (MSEL), a standardized instrument

to assess cognitive development[69], was also administered. An algorithm based on ADOS and MSEL scores previously published by the Baby Siblings Research Consortium[70] was used to classify children with typically developing (TD), Non-TD, or ASD outcomes. Child outcome at 36-months was used in the present study. Children with ASD outcomes (*n*= 70) had scores over the ADOS cutoff and met the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for ASD. Participants with Non-TD outcomes (*n*=37) had scores within three points of the ADOS cutoff and/or MSEL scores 1.5 to 2 standard deviants below average.[70] The remaining children were classified as TD (*n*=154).

Exposure assessment

Quantification of Perceived Stress

MPS in this study is defined as psychosocial stress measured using the Perceived Stress Scale (PSS) questionnaire designed by Cohen & Williamson.[86] This questionnaire was given to mothers at each prenatal visit. The PSS was specifically designed to be generalizable among community samples from various subpopulations of varying socioeconomic statuses[86] and comprehensible to anyone having at least a junior high education level. Language of the questionnaire is general to avoid content specific to any subpopulation. The scale assesses the participant's perceived stress over the previous month. It includes ten questions that tap into the general stress in the participant's life that are outside of their control. For example, a couple of questions ask: "*In the last month, how often have you been upset because of something that happened unexpectedly?"*; or "*In the last month, how often have you found that you could not cope with all the things that you had to do?".* Responses range from 0 (Not at all) to 4 (Very often). A higher PSS total score indicates higher perceived stress.

Depending on how far along a mother was during her pregnancy, MARBLES conducted up to two visits per trimester for a total of up to six visits throughout pregnancy. Thus, each mother had up to six prenatal perceived stress scores during the time she was pregnant in the study.

Measures of perceived stress

Because mothers have up to three PSS scores each trimester, an average PSS total score for each trimester was calculated for each mother by summing all available PSS scores in the trimester and dividing by number of scores available for that trimester. A grand total average for each mother was calculated by summing all responses throughout pregnancy and dividing by available scores throughout pregnancy.

PSS Deviations

PSS average scores for each trimester were first analyzed to examine whether baseline stress level for pregnancy was associated with ASD. Then, PSS average scores for each trimester were analyzed by creating new variables indicating the deviations of the trimester average scores from the grand total average score. The rationale for using the deviations from the total average as opposed to the trimester averages alone was because this allowed for consideration of the fact that some mothers may have higher baseline stress than other mothers in the study, and that it is this difference from her baseline stress during pregnancy that could be important. Without looking at deviations, analyses would assume that the differences between mothers were the same as the differences within each individual mother. However, recognizing that there may be differences within each mother as well as between all mothers, using the deviation variables with the total pregnancy averages allowed for consideration of these differences.

Confounding Assessment

Covariates were selected *a priori* based on the Directed Acyclic Graph (DAG) in Figure 1. This DAG shows bold red arrows signifying known associations in populations at high risk for ASD (i.e., siblings of children with ASD). The primary model with covariates selected based on the DAG has the advantage of adjusting for a sufficient set of confounders based on subject matter expertise in known high familial risk

populations. All backdoor paths were identified based on these known associations. To block all backdoor paths, the minimally sufficient set that the primary analysis controlled for included: maternal race/ethnicity, maternal age, and home ownership as a proxy for socioeconomic status.

A sensitivity analysis was conducted, which considered additional potential confounding variables based on known associations in general population ASD studies. All backdoor paths were identified, and the minimally sufficient set to block these paths included the following variables in addition to those in the primary analysis: immune stressors during pregnancy, maternal country of birth (Inside or Outside the US), season of conception, parity, cotinine, air pollution compounds (ambient carbon monoxide (CO), nitrogen oxide (NO), nitrogen dioxide (NO2), ozone – 24hr (O3), adjusted PM10 (PM10a), and PM2.5), and other chemicals (monoethyl phthalate (MEP), bisphenol A (BPA), and ethylparaben (EtPB)). However, a number of these covariates were measured in only a subset of participants and so were removed to avoid overfitting. Thus, the following covariates were included in the sensitivity analysis for: Trimester 1 – maternal race/ethnicity, maternal age, home ownership, maternal country of birth, and parity; Trimesters 2 and 3 – maternal race/ethnicity, maternal age, home ownership, maternal country of birth, parity, CO, NO, NO2, O3, PM10a, and PM2.5. The maternal psychiatric conditions variable was not included in analyses due to unavailability of these data.

Below is information on how data on variables were collected.

Race and ethnicity were collected from participants through multiple sources, which included the Environmental Exposure Questionnaire phone interview, the Family Information Form, the MARBLES database tracking system, and California birth files. Responses from four sources were self-reported by the participant. To determine race, participants were asked if they identified as: white, Black/African American, American Indian/Alaska Native, Asian, Native Hawaiian/Other Pacific Islander, Other, or more than one race. To determine ethnicity, participants were asked if they identified as Hispanic or not

Hispanic. Because this information was collected multiple times, if responses did not align, MARBLES programmers manually reviewed participants to determine any errors and correctly classified race/ethnicity. Due to small sample size and low frequencies in the African American, Hispanic, American Indian, and mixed race groups, race and ethnicity were collapsed into three categories: non-Hispanic white, Asian, and Other.

Home ownership information was used as an indirect measure for SES and was collected through the MARBLES Environmental Exposure Questionnaire (EEQ).

Immune stressors were comprised of any infections mothers may have had during pregnancy. This information was collected through the EEQ. Mothers reported, if any, which months they had the following infections: chicken pox, flu, measles, mumps, rubella, shingles, gonorrhea, syphilis, toxoplasmosis, cytomegalovirus, hepatitis, genital herpes, urinary tract infection, pelvic inflammatory disease (PID), chlamydia, trichomonas, bacterial vaginosis, vulvoginal yeast, sinusitis, bronchitis, tuberculosis, Lyme's disease, other vaginal infections (asked to specify), other respiratory infections besides a cold (asked to specify type), and any other infections (asked to specify type). An immune risk score was calculated for each participant for each trimester, where infections were totaled for that trimester. Because there was not enough variability for a robust estimation when considering trimesters separately, immune stressors were collapsed into a dichotomous variable of "0" or "1+" during all of pregnancy.

Maternal country of birth was collected through CA Birth Files of the child. If not stated in the birth files, the EEQ phone interview was used, where mothers reported their place of birth. Responses were coded to indicate if mothers were born inside or outside of the United States.

Parity was collected through the EEQ. Any pregnancies over 20 weeks gestation prior to the child of interest's birth were considered, no matter if the pregnancy resulted in miscarriage, abortion, still at birth, or live birth. Categories were collapsed to "1" or "2+" to allow variability for a more robust estimation.

Maternal age at delivery was calculated using maternal date of birth and child's date of birth.

Environmental exposures were comprised of smoking information (cotinine), air pollution, and chemical exposures. Cotinine was calculated from prenatal maternal urine samples with the limit of detection being 0.2 ng/mL. Because cotinine exposure was measured in only a small subset of women, this variable was dropped from the sensitivity analysis.

Air pollution information, namely ambient CO, NO, NO2, O3, PM2.5 and adjusted PM10 air pollution monthly exposure, were calculated based on residential history collected for all participants. The pregnancy exposures were estimated from data downloaded from the U.S. Environmental Protection Agency's (EPA) Air Quality System (AQS) database [87] that provides daily average air pollution concentrations which are spatially interpolated to residential addresses for each participant. Monthly averages were calculated from the daily averages, and these daily averages were spatially interpolated from the air quality monitoring stations' locations to the residence locations using inverse distance-squared weighting. An adjusted estimate for PM10 was equal to PM2.5 when the originally estimated PM10 was less than the corresponding PM2.5 and the distance to the nearest monitoring station was shorter for the PM2.5 estimate than the original PM10 estimate.

Chemical exposures were selected to test for confounding based on findings from previous MARBLES investigations of environmental exposures.[88,89] Chemicals examined for possible confounding were: the phthalate (metabolite) MEP,[88] the phenol BPA,[89] and the paraben EtPB.[89] Methods for determining

exposure based on concentrations in urine have been previously published.[88,89] In short, up to four urine samples were collected from each mother in the second and third trimesters of pregnancy. Specific gravity-corrected averages for each trimester were calculated and used for confounding assessment. Because MEP, BPA, and EtPB exposures were measured in only a small subset of women, these variables were dropped from the sensitivity analysis.

Statistical Analysis

Using the primary model, multinomial logistic regressions were fitted for neurodevelopmental outcomes for each of the three trimesters' average PSS scores separately, controlling for the covariates above, to explore baseline PSS score and outcome. Average PSS scores per trimester were used for the exposure variable in these regressions. Because the prevalence of ASD in males is higher than females[52] and there is biologic plausibility,[53,54] sex-specific effects were explored by adding an interaction term between average PSS scores and child sex in each trimesters' regression models.

Multinomial logistic regressions were also fitted for the deviations for each trimester separately, controlling for the covariates listed above, to examine the association of PSS score deviations and outcome.

Lastly, multinomial logistic regressions fitted for perceived stress for each trimester and neurodevelopmental outcome were stratified by race (non-Hispanic white, Asian, and Other). One adjusted model for each trimester was fitted, which included an interaction term for average PSS score and maternal race/ethnicity. To more strongly confirm the associations, three separate adjusted models were fitted for each trimester for the three race groups separately.

All multinomial logistic regressions were then fitted for the sensitivity analysis. All statistical analyses were performed with SAS version 9.4 (Institute Inc. Carny, NC, USA). P-values ≤ 0.05 were considered statistically significant.

RESULTS

This study consisted of 261 mother-child pairs. In total, mothers were primarily non-Hispanic white homeowners that were born in the U.S. Most mothers did not report experiencing any immune stressors or infections during pregnancy. Season of conception was nearly evenly distributed amongst the four seasons. All mothers had one previous pregnancy before the current pregnancy of interest for this study. Mean age was 34.6 years. Mean levels of environmental exposures are reported in **Table 1**. Distributions of characteristics by neurodevelopmental outcome are also included in **Table 1**.

Trimester average PSS scores were significantly associated with Non-TD in the first trimester (**Table 2**). A one point increase in PSS score was associated with 10% increased relative risk in Non-TD compared to TD (RRR 1.10, 95% CI (1.00, 1.21)). Findings with Non-TD were in the same direction, but attenuated and not significant in the second and third trimesters (**Table 2**).

A one point increase in PSS score was significantly associated with 8% increased relative risk of ASD in trimester 2 (RRR 1.08, 95% CI (1.02, 1.14)) and 8% increased relative risk of ASD in trimester 3 (RRR 1.08, 95% CI (1.03, 1.14)). Findings with the ASD group were in the same direction, but attenuated and not significant in the first trimester (**Table 2**).

When examining potential effect modification by child sex on the association of average PSS score and neurodevelopmental outcome, differences between female and male relative risk ratios of outcome were not meaningfully different in any trimester (**Table 3**).

Associations between trimester deviations and neurodevelopmental outcome were non-significant for both Non-TD and ASD (**Table 4**).

When stratified by self-reported race and ethnicity, findings were not meaningfully different when comparing relative risk for Asian and Other race groups to non-Hispanic white (**Table 5**). When validating the interaction model with the separate race group stratification models, the RRRs were similar (**Tables 6 and 7**).

In the sensitivity analysis, relative risk ratios were generally higher for all multinomial logistic regressions (**Tables 8-11**). In trimester 3, a one point increase in PSS score was associated with 13% increase relative risk in ASD compared to TD (RRR 1.13, 95% CI (1.03, 1.24)), which is 5% higher risk than in the primary model (**Table 8**).

DISCUSSION

This is the first study to examine and find an association between prenatal perceived stress and ASD and Non-TD outcome in a high familial risk population.

Novel to this study is the measurement of MPS by perceived stress. Previous investigations have measured MPS as stressful life events, such as deaths in the family, family discord, rocket attacks, and ice storms.[21,24,26,36] Findings on the associations of these stressful life events with ASD risk were mixed, with some showing no association and others showing a positive association. The authors of the present study examined perceived stress, with the rationale being that it may not be the stressful life events experienced themselves that could be associated with an increased risk to the child, but instead how the mother perceives these events. Additionally, there are also everyday stressors that are not considered when solely focusing on stressful life events. For example, everyday work stressors or the holiday season are not something that would be considered a major stressful life event but undoubtedly have an effect

on a person. Perceived stress is a way to measure these non-major life events that still result in stress on the mother. Thus, the perceived stress score was used, and these significant findings confirm the hypothesis that perception of stress is a risk factor, whereas previous work suggests stressful events themselves are not.

All associations with trimester average PSS scores were significant across trimesters for ASD, except in the first trimester. This finding and the result that perceived stress had the biggest increased relative risk in the second and third trimesters are similar to previous investigations' findings indicating that this time period of pregnancy is associated with the greatest risk.[22,23,90] Although the mechanisms behind any associations between MPS and neurodevelopment warrant future research, the timing for a critical period for stress in the second half of pregnancy is in line with a common theory of the way maternal stress affects the fetus through the hypothalamic-pituitary-adrenal (HPA) axis. While the fetal hypothalamus begins forming around 9-10 weeks gestation, it is not fully able to function until early in the second trimester of pregnancy.[44] It is theorized that during the second trimester, a mother's prenatal stress may begin to be experienced by the fetus, which could alter the fetus's HPA axis function to a higher set point or greater reactivity, which in turn suppresses the fetus's immune response, resulting in atypical development.[47] Once the HPA axis is developed, the fetus would experience the effects of the mother's stress in the second and third trimester.

Analyses with trimester deviation scores were not significant. This provides evidence against the hypothesis that change in stress from each individual mother's baseline is a risk factor, and instead indicates that cross-individual differences in baseline or chronic stress (and their perception of it) could be more relevant to the child's neurodevelopmental outcomes.

When results were stratified by self-reported race/ethnicity, relative risks varied in relation to the study population as a whole, with the non-Hispanic white group having elevated relative risk in some

trimesters and Asian and Other having elevated relative risk in other trimesters. These findings warrant more exploration as it is unclear if these associations still hold if the sample size is increased given unstable estimates in the Asian and Other race/ethnicity groups. It is important to note that relative risk ratios did not differ much across races for each trimester. Though not statistically different across races, one explanation of why estimates might differ by race is that non-white races experience compounding stressors due to factors such as racism, discrimination, microaggressions, stressors of assimilating, and other added stressors that white mothers may not experience. Thus, their compounding stress in non-white races might be more strongly associated with ASD or Non-TD outcome. This study was not able to explore these racial factors due to sample size constraints. Future, larger studies should strive to enroll non-white ethnic and racial groups, as most existing ASD literature focuses on white children. Focusing on these non-white groups that are often disproportionately impacted will allow a paradigm shift of moving away from an overarching treatment method for ASD and instead shift toward equitable and culturally sensitive options that take into consideration each groups' differences and needs.

When comparing the primary model to the sensitivity analysis, relative risk generally increased for all associations. Findings were in the same direction as the primary model, but it is worth noting that precision was lower. This could be due to missing data for some of the added covariates in the sensitivity analysis since some variables were measured in only a subset of participants. MARBLES should aim to collect this information for all participants to conduct a more precise analysis.

A major strength of this study is that it used prospective measures from a longitudinal cohort study. Most research on stress and ASD has been done retrospectively. Following the MARBLES cohort prospectively allows for a better understanding of timing of exposure and outcome. Another strength includes child neurodevelopmental assessments by trained experts using gold-standard clinical methods to diagnose ASD. Additionally, since an important criterion of being eligible for participation in

MARBLES, a high familial risk cohort, required that mothers have at least one child already diagnosed with ASD, MARBLES adhered to meticulous protocols to ensure that the proband has received a formal diagnosis. If scores from their diagnosis could not be obtained, study clinicians performed an assessment in-clinic to confirm the diagnosis.

A limitation of this research is that recruitment lists were generated from cases of autism reported from DDS, meaning that any children with autism that were not referred to a regional center to attain services may not have been included in these DDS lists. This may have included children that were previously diagnosed but parents did not move forward with contacting a regional center. Or, this also may have included children with milder cases of autism whom do not have a diagnosis. These families would not have been on the list of eligible families in DDS that were contacted during recruitment. However, one study found that 75-80% of children with autism are enrolled with DDS and concluded that the characteristics of children not enrolled with DDS would have to be dramatically different from the enrolled children to have substantially different findings.[91] Additionally, MARBLES also recruited families who were referred by outside providers, by word of mouth, outreach events, or other research studies. This may have helped in recruitment of families that did not received services with DDS.

This high familial risk pregnancy cohort has a limitation of generalizability of findings. However, some associations with risk and protective factors found in general populations have been replicated in high-risk families.[92,93] Further research could conduct a sensitivity analysis to see if overall average PSS scores in this high-risk pregnancy cohort may be similar to that of women of childbearing age in another study.

The findings of this study suggest that stress reduction intervention could serve as preventative measures that help optimize the child's long term neurodevelopmental health in high familial risk families. Larger studies are needed to validate and replicate these findings in other populations. Further studies are needed to explore differences of race/ethnicity and the impact structural racism might play

in these differences. Future studies should also look at biomarkers for stress in association with neurodevelopmental outcome.

APPENDIX

Figure 1. Directed Acyclic Graph (DAG)

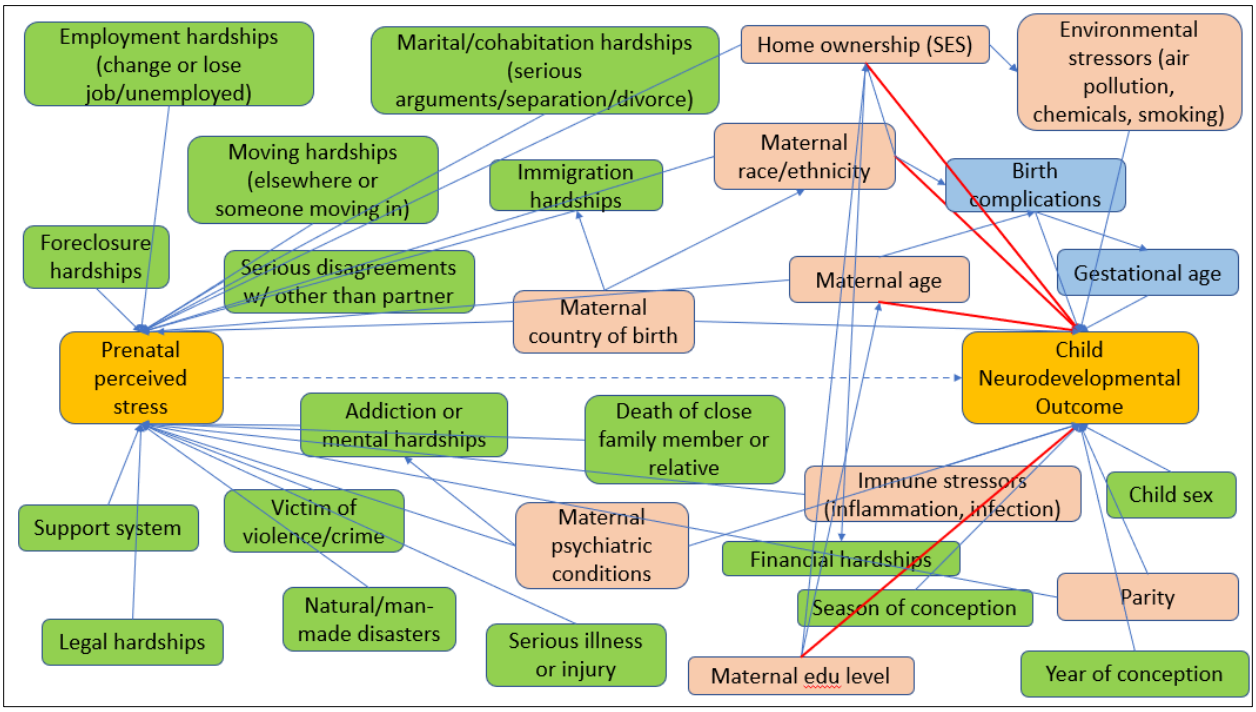

Figure 1. Directed Acyclic Graph created to select covariates to control for a priori. Exposure variable is prenatal perceived stress. Outcome is child neurodevelopmental outcome. Variables in green are upstream of the exposure variable. Variables in blue are on the pathway from exposure to outcome or are downstream of outcome. Variables in red are confounders. Blue arrows show associations of variables from existing literature in general population studies. Bold red arrows show associations of variables from existing literature in high familial risk populations. Variables selected as the minimally sufficient set to block all backdoor paths in the primary analysis were: maternal race/ethnicity, maternal age, and home ownership as a proxy for socioeconomic status (SES). Variables selected as the minimally sufficient set in addition to the primary analysis variables were: maternal psychiatric conditions, immune stressors, maternal country of birth, parity, cotinine, ambient carbon monoxide, nitrogen oxide, nitrogen dioxide, ozone – 24hr, adjusted PM10, PM 2.5, monoethyl phthalate, Bisphenol A, and ethylparaben).

Table 1. Characteristics of study participants, stratified by child neurodevelopmental outcome

Characteristics	Row total		TD		ASD		Non-TD		*p*-value
	n	%	n	%	n	%	n	%	
Mother Race/Ethnicity									0.47
Non-Hispanic White	129	49.43	79	51.3	32	45.71	18	48.65	
Hispanic	52	19.92	30	19.48	14	20	8	21.62	
Black/African American	14	5.36	4	2.6	7	10	3	8.11	
Asian	49	18.77	31	20.13	11	15.71	7	18.92	
Mixed Race/Other	17	6.51	10	6.49	6	8.57	1	2.7	
Homeowner									0.14
No	112	44.27	60	39.47	35	53.85	17	47.22	
Yes	141	55.73	92	60.53	30	46.15	19	52.78	
Immune Stressors									
Trimester 1									0.7
0	259	99.23	153	99.35	69	98.57	37	100	
1+	2	0.77	1	0.65	1	1.43	0	0	
Trimester 2									0.42
0	251	96.17	147	95.45	67	95.71	37	100	
1+	10	3.83	7	4.55	3	4.29	0	0	
Trimester 3									0.39
0	239	91.57	140	90.91	63	90	36	97.3	
1+	22	8.43	14	9.09	7	10	1	2.7	
Maternal Country of Birth									0.99
Inside the U.S.	182	71.94	110	71.9	45	71.43	27	72.97	
Outside the U.S.	71	28.06	43	28.1	18	28.57	10	27.03	
Season of Conception									0.73
Fall	77	29.5	40	25.97	23	32.86	14	37.84	
Winter	57	31.84	35	22.73	15	21.43	7	18.92	
Spring	67	25.67	39	25.32	18	25.71	10	27.03	
Summer	60	22.99	40	25.97	14	20	6	16.22	

Parity													0.89
1	106	66.67		70	67.96		22	64.71		14	63.64		
2+	53	33.33		33	32.04		12	35.29		8	36.36		
	Total			**TD**			**ASD**			**Non-TD**			***p*-**
	n	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	**value**
Maternal Age (years)	261	34.61	4.76	154	34.98	4.86	70	34.32	4.67	37	33.59	4.4	0.24
Cotinine (ng/mL)	148	26.5	150.69	89	12.93	94.7	39	70.73	253.54	20	0.64	1.01	0.39
Carbon Monoxide (ppb)	206	0.35	0.08	120	0.34	0.08	57	0.34	0.08	29	0.37	0.09	0.21
Nitrogen Oxide (ppb)	208	6.92	3.58	121	6.92	3.72	58	6.71	3.51	29	7.37	3.2	0.44
Nitrogen Dioxide (ppb)	208	10.27	2.63	121	10.4	2.84	58	10.04	2.49	29	10.18	1.94	0.67
Ozone - 24hr (ppb)	209	24.74	3.93	122	24.79	4.25	58	24.62	3.41	29	24.77	3.63	0.98
Adjusted PM10 (µg/m3)	208	19.44	4.25	121	19.86	4.6	58	18.3	3.74	29	19.93	3.27	0.06
PM2.5 (µg/m3)	209	9.82	2.27	122	9.83	2.43	58	9.55	1.97	29	10.27	2.1	0.38
Mono-ethyl phthalate (MEP) (µg/L)													
Trimester 2	101	3.34	0.98	61	3.33	0.99	26	3.3	0.94	14	3.46	1.06	0.88
Trimester 3	142	60.32	270.96	83	34.24	37.3	39	44.33	61.48	20	199.71	712.29	0.43
Bisphenol A (BPA) (ng/L)													
Trimester 2	110	1.53	1.14	67	1.51	1.09	28	1.41	0.99	15	1.89	1.6	0.77
Trimester 3	154	1.92	2.04	88	2.06	2.37	43	1.68	1.35	23	1.81	1.77	0.79
Ethylparaben (EtPB) (ng/L)													
Trimester 2	110	12.55	54.89	67	9.31	17.92	28	25.48	105.47	15	2.88	2.89	0.84
Trimester 3	154	8.64	22.88	88	9.97	28.5	43	5.93	9.58	23	8.6	15.27	0.97

Table 1. Characteristics of MARBLES mothers included in the analyses by neurodevelopmental outcome group. Immune stressors were determined from calculating a risk score based on how may infections mothers reported during each trimester of pregnancy. Infections recorded are listed in the methods section. P-values were obtained from chi-square tests. Note: TD=typically developing, Non-TD=nontypically developing, ASD=autism spectrum disorder.

Table 2. Results of average trimester PSS score and neurodevelopmental outcome

Trimester PSS average scores						
	ASD (vs. TD)			Non-TD (vs. TD)		
Trimester (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**
Trimester 1 (n=95)	27	1.04 (0.96, 1.12)	0.36	14	1.10 (1.00, 1.21)	0.04
Trimester 2 (n=181)	47	1.08 (1.02, 1.14)	0.01	24	1.06 (0.99, 1.14)	0.11
Trimester 3 (n=241)	61	1.08 (1.03, 1.14)	0.003	33	1.06 (0.99, 1.13)	0.11

Table 2. Results from multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and Non-TD groups, with TD as the reference group.

Table 3. Results of sex-specific estimates of neurodevelopmental outcome for PSS score in ASD vs. TD

Female							
	ASD (vs. TD)			Non-TD (vs. TD)			
Trimester (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**Interaction p-value**
Trimester 1 (n=45)	9	1.00 (0.92, 1.10)	0.96	7	1.10 (0.98, 1.23)	0.11	
Trimester 2 (n=78)	14	1.04 (0.98, 1.12)	0.18	11	1.06 (0.98, 1.15)	0.16	REF
Trimester 3 (n=105)	18	1.06 (1.00, 1.13)	0.04	17	1.06 (0.99, 1.14)	0.07	
Male							
	ASD (vs. TD)			Non-TD (vs. TD)			
Trimester (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**Interaction p-value**
Trimester 1 (n=50)	18	1.06 (0.97, 1.15)	0.19	7	1.12 (1.01, 1.24)	0.03	0.3
Trimester 2 (n=103)	33	1.10 (1.03, 1.16)	0.003	13	1.06 (0.98, 1.15)	0.12	0.15
Trimester 3 (n=136)	43	1.10 (1.04, 1.16)	0.0009	16	1.04 (0.97, 1.12)	0.23	0.12

Table 3. Results from adjusted multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates to compare ASD vs. TD with an interaction term for average PSS score and child sex. Total n's ,sex-specific relative risk ratios, 95% confidence intervals, and p-values are presented. RRRs were calculated from one model, which included an interaction term for PSS score and child sex.

Table 4. Results of trimester PSS score deviations and neurodevelopmental outcome

Trimester deviations						
	ASD (vs. TD)			Non-TD (vs. TD)		
Trimester (total n)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value
Trimester 1 (n=95)	27	0.89 (0.72, 1.10)	0.29	14	1.09 (0.88, 1.35)	0.44
Trimester 2 (n=181)	47	0.96 (0.77, 1.19)	0.69	24	1.05 (0.80, 1.36)	0.74
Trimester 3 (n=241)	61	0.99 (0.83, 1.18)	0.92	33	0.87 (0.68, 1.12)	0.28

Table 4. Results from multinomial logistic regressions of deviation scores in each trimester, adjusted for covariates. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and Non-TD groups, with TD as the reference group.

Table 5. Results of race-specific estimates of neurodevelopmental outcome for PSS score in ASD vs. TD

ASD (vs. TD)									
	non-Hispanic white			Asian			Other			Interaction p-value
Trimester	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	
Trimester 1	15	1.07 (0.96, 1.19)	0.24	6	1.01 (0.79, 1.28)	0.95	6	0.99 (0.87, 1.14)	0.94	0.77
Trimester 2	26	1.08 (1.00, 1.17)	0.04	7	1.07 (0.92, 1.25)	0.4	14	1.07 (0.96, 1.18)	0.21	0.97
Trimester 3	30	1.06 (0.99, 1.14)	0.11	9	1.03 (0.90, 1.18)	0.65	22	1.16 (1.04, 1.29)	0.005	0.62
Non-TD (vs. TD)										
	non-Hispanic white			Asian			Other			Interaction p-value
Trimester	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	
Trimester 1	6	1.07 (0.93, 1.23)	0.32	5	1.18 (0.93, 1.50)	0.17	3	1.13 (0.94, 1.36)	0.2	0.77
Trimester 2	14	1.08 (0.98, 1.19)	0.11	5	1.06 (0.89, 1.27)	0.52	5	1.01 (0.87, 1.18)	0.86	0.97
Trimester 3	16	1.04 (0.95, 1.14)	0.44	7	1.04 (0.89, 1.21)	0.63	10	1.10 (0.97, 1.25)	0.13	0.62

Table 5. Results from adjusted multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates to compare ASD vs. TD and Non-TD vs. TD. Total n's, race-specific relative risk ratios, 95% confidence intervals, and p-values are presented. RRRs were calculated from one model, which included an interaction term for PSS score and maternal race.

Table 6. Results of trimester average PSS scores and ASD outcome stratified by race

ASD (vs. TD)									
	non-Hispanic white			Asian			Other		
Trimester	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value
Trimester 1	15	1.06 (0.95, 1.19)	0.3	6	0.94 (0.71, 1.22)	0.63	6	0.99 (0.86, 1.13)	0.84
Trimester 2	26	1.08 (1.00, 1.17)	0.04	7	1.05 (0.89, 1.23)	0.56	14	1.06 (0.95, 1.18)	0.31
Trimester 3	30	1.06 (0.99, 1.14)	0.11	9	1.07 (0.92, 1.23)	0.39	22	1.16 (1.04, 1.29)	0.007

Table 6. Results from multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates, and stratified by race to compare ASD and TD. Total n's, relative risk ratios, 95% confidence intervals, and p-values compare ASD group and TD as the reference group across non-Hispanic white, Asian, and other races. RRRs were calculated from three separate models to stratify for the three race groups.

Table 7. Results of trimester average PSS scores and Non-TD outcome stratified by race

Non-TD (vs. TD)									
	non-Hispanic white			Asian			Other		
Trimester	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value
Trimester 1	6	1.06 (0.92, 1.22)	0.41	5	1.28 (0.92, 1.77)	0.14	3	1.16 (0.96, 1.40)	0.14
Trimester 2	14	1.08 (0.98, 1.19)	0.11	5	1.06 (0.88, 1.27)	0.57	5	1.03 (0.88, 1.20)	0.74
Trimester 3	16	1.04 (0.95, 1.14)	0.43	7	1.04 (0.88, 1.21)	0.67	10	1.10 (0.98, 1.25)	0.12

Table 7. Results from multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates, and stratified by race to compare Non-TD and TD. Total n's, relative risk ratios, 95% confidence intervals, and p-values compare Non-TD and TD as the reference group across non-Hispanic white, Asian, and other races. RRRs were calculated from three separate models to stratify for the three race groups.

Table 8. Results of sensitivity analysis— average trimester PSS score and neurodevelopmental outcome

Trimester PSS average scores						
	ASD (vs. TD)			Non-TD (vs. TD)		
Trimester (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**
Trimester 1 (n=61)	14	1.05 (0.93, 1.19)	0.42	8	1.25 (0.99, 1.58)	0.06
Trimester 2 (n=89)	19	1.11 (1.00, 1.24)	0.05	13	1.10 (0.98, 1.25)	0.12
Trimester 3 (n=122)	24	1.13 (1.03, 1.24)	0.01	18	1.14 (1.03, 1.27)	0.01

Table 8. Results from multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates in the sensitivity analysis. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and Non-TD groups, with TD as the reference group.

Table 9. Results of sensitivity analysis— sex-specific estimates of neurodevelopmental outcome for PSS score in ASD vs. TD

Female							
	ASD (vs. TD)			Non-TD (vs. TD)			Interaction p-value
Trimester (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	
Trimester 1 (n=30)	4	0.99 (0.86, 1.15)	0.92	4	1.23 (0.95, 1.58)	0.12	REF
Trimester 2 (n=54)	7	1.06 (0.94, 1.22)	0.33	7	1.10 (0.96, 1.26)	0.17	
Trimester 3 (n=69)	9	1.10 (0.99, 1.22)	0.07	9	1.14 (1.02, 1.28)	0.03	
Male							
	ASD (vs. TD)			Non-TD (vs. TD)			Interaction p-value
Trimester (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	
Trimester 1 (n=31)	10	1.10 (0.95, 1.27)	0.21	4	1.28 (0.98, 1.66)	0.07	0.24
Trimester 2 (n=60)	17	1.13 (1.01, 1.26)	0.04	8	1.10 (0.97, 1.25)	0.15	0.48
Trimester 3 (n=83)	20	1.14 (1.04, 1.26)	0.007	12	1.14 (1.02, 1.27)	0.02	0.53

Table 9. Results from adjusted multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates in the sensitivity analysis to compare ASD vs. TD with an interaction term for average PSS score and child sex. Total n's ,sex-specific relative risk ratios, 95% confidence intervals, and p-values are presented. RRRs were calculated from one model, which included an interaction term for PSS score and child sex.

Table 10. Results of sensitivity analysis— trimester PSS score deviations and neurodevelopmental outcome

Trimester deviations						
	ASD (vs. TD)			Non-TD (vs. TD)		
Trimester (total n)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value
Trimester 1 (n=61)	14	0.92 (0.68, 1.23)	0.56	8	1.00 (0.58, 1.71)	0.99
Trimester 2 (n=89)	19	1.00 (0.68, 1.47)	0.98	13	0.87 (0.51, 1.49)	0.61
Trimester 3 (n=122)	24	0.99 (0.73, 1.34)	0.95	18	0.82 (0.56, 1.20)	0.31

Table 10. Results from multinomial logistic regressions of deviation scores in each trimester, adjusted for covariates in the sensitivity analysis. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and Non-TD groups, with TD as the reference group.

Table 11. Results of sensitivity analysis—race-specific estimates of neurodevelopmental outcome for PSS score in ASD vs. TD

ASD (vs. TD)									
	non-Hispanic white			Asian			Other			Interaction p-value
Trimester	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	
Trimester 1	8	1.17 (0.92, 1.49)	0.2	2	0.94 (0.62, 1.44)	0.78	4	0.98 (0.82, 1.19)	0.85	0.58
Trimester 2	10	1.18 (1.02, 1.37)	0.03	3	1.10 (0.85, 1.42)	0.48	6	1.01 (0.82, 1.25)	0.89	0.19
Trimester 3	12	1.16 (1.01, 1.33)	0.03	3	1.04 (0.85, 1.27)	0.72	9	1.13 (0.97, 1.31)	0.11	0.89
Non-TD (vs. TD)										
	non-Hispanic white			Asian			Other			Interaction p-value
Trimester	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	
Trimester 1	2	1.93 (0.26, 14.11)	0.52	4	1.44 (0.98, 2.13)	0.07	2	0.98 (0.66, 1.47)	0.92	0.58
Trimester 2	7	1.25 (1.03, 1.50)	0.02	3	1.19 (0.85, 1.67)	0.31	3	0.84 (0.66, 1.07)	0.16	0.19
Trimester 3	7	1.19 (1.006, 1.40)	0.04	4	1.09 (0.89, 1.35)	0.39	7	1.12 (0.95, 1.31)	0.18	0.89

Table 11. Results from adjusted multinomial logistic regressions of averaged perceived stress scale scores in each trimester, adjusted for covariates in the sensitivity analysis to compare ASD vs. TD and Non-TD vs. TD. Total n's, race-specific relative risk ratios, 95% confidence intervals, and p-values are presented. RRRs were calculated from one model, which included an interaction term for PSS score and maternal race.

Chapter 3

Prenatal urinary cortisol as a risk factor for ASD and non-typical developmental outcome in MARBLES: A high familial risk cohort

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ABSTRACT

Background and Objective: Investigations examining the association between prenatal stress and ASD in the child have had varying findings. Prenatal stress is a complex exposure that has been measured in various ways, including stressful life events, perceived stress, and various biomarker. Measuring stress biomarkers can indicate changes to the body caused by specific stressors, identify people at risk for development of disorders, and help in creating interventions for stress management or reduction. Cortisol is the body's main stress hormone and is the most common biological measure of stress. One investigation to date has prospectively studied fetal cortisol exposure by measuring maternal plasma cortisol at 15, 19, 25, 31, and 37 weeks and later assessing its association with child ASD symptoms at age 5 through the Social Communication Questionnaire, finding that fetal exposure to lower levels of maternal cortisol was associated with higher levels of ASD symptoms in boys. The present study prospectively examined the association between maternal urinary cortisol during pregnancy and subsequent diagnosed ASD and non-typical development outcomes, where expert clinicians assessed and confirmed the diagnosis of the child. This is also the first study to examine recurrence risk in a high familial risk population where mothers have already had at least one child with ASD and thus their subsequent children are at elevated risk of developing ASD as well.

Methods: This study included 146 women from the prospective longitudinal Markers of Autism Risk in Babies: Learning Early Signs (MARBLES) cohort. Cortisol assessment was measured through 24-hr urine collections that mothers provided once per trimester. The 24-hr samples were assayed to provide cortisol measurements and creatinine values. Child neurodevelopment was assessed longitudinally from birth through three years of age by trained psychologists at the UC Davis MIND Institute who administered the Autism Diagnostic Observation Schedule (ADOS) and Mullen Scales of Early Learning (MSEL). The Baby Siblings Research Consortium (BSRC) algorithm based on ADOS and MSEL scores was used to classify children with ASD, non-typically developing (Non-TD), or typically developing (TD)

outcomes at three years of age. Multinomial logistic regressions were fitted for average natural log transformed cortisol concentrations each trimester as predictors of ASD/Non-TD outcome, controlling for maternal race/ethnicity, maternal age, home ownership, number of thaws in the sample, and creatinine level. Multinomial logistic regressions also tested the association of cortisol level deviations each trimester from each mother's average with ASD/Non-TD outcome, controlling for the same covariates above. Additionally, multinomial logistic regressions examined the association of cortisol scores stratified by maternal race (non-Hispanic white and Other). Lastly, receiver operating characteristic (ROC) curves were graphed to compare the predictability of ASD outcome using perceived stress and cortisol as separate stress exposures of interest.

Results: All regressions resulted in non-significant findings, but the direction of results were consistent with previous findings that decreased cortisol levels were associated with higher ASD risk. Decreased cortisol exposure during pregnancy and elevated risk of ASD could be explained by mothers having a decreased reaction to stressors due to chronic stress associated with already caring for a child with ASD. Chronic stress can alter HPA-axis function so that a mother's cortisol level change resulting from other stressors might be attenuated. From the ROC curves, perceived stress was a better predictor of ASD outcome with a greater area under the curve (AUC) of 0.66, 95% CI (0.58, 0.74)) compared to cortisol (AUC = 0.54, 95% CI (0.43, 0.68)). Both measures of stress were fitted into one model to graph paired ROC curves in order to compare the two curves statistically. Results showed that they were not meaningfully different (p-value 0.14).

Conclusion: This study did not find an association between prenatal cortisol and neurodevelopmental outcome, but adds to the growing knowledge of prenatal cortisol output as a risk factor for ASD or non-TD outcomes in the child. Larger sample sizes, particularly recruiting mothers of diverse race backgrounds, are needed to further investigate these associations and detect meaningful effects. In

addition to considering cortisol as a stress biomarker, and future research is also needed to understand associations between stress exposures and cortisol output.

BACKGROUND

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by restricted or repetitive behaviors and challenges with social communication and interaction.[94] The CDC most recently reported that 1 in 44 children in the US have ASD.[95] With the prevalence continually increasing, ASD continues to be a pressing public health concern. Part of the explanation of this increase in ASD is due to changes in diagnostic criteria, earlier age of diagnosis, and the diagnosis of milder cases,[83] but this leaves room for speculation regarding what other factors could be contributing to the striking increase. The etiology of ASD remains unknown, but research in the fields of genetics and environmental exposures are developing as more and more evidence points towards a combination of genes and the environment causing the condition.

Research on environmental exposures has included prenatal stress, which has been found to be associated with greater risk of ASD. Prenatal stress is a complex exposure that has been measured in various ways, including stressful life events,[21,24,26,35,36] perceived stress, and various biomarkers, like blood vitamin metabolite levels, glutathione peroxidase, and methionine.[38] Measuring biomarkers of stress is important because it can help indicate changes to the body caused by specific stressors and help in creating interventions for management or reduction of stress, thus reducing risk of developing stress-related disorders.[39]

Cortisol is a glucocorticoid that plays an important role in the development of the fetal brain and other organs. The hypothalamic-pituitary-adrenal (HPA) axis is one of the body's primary stress-response systems, and fetal exposure to glucocorticoids affects programming of the HPA axis.[33] Corticotropin-releasing hormone (CRH) is synthesized from the hypothalamic paraventricular nucleus, which then stimulates the release of adrenocorticotropic hormone (ACTH). ACTH promotes the production of cortisol from the adrenal cortex and feeds back to modulate HPA activity.[40] In pregnancy, CRH is

synthesized from the placenta and changes the regulation of the maternal HPA axis.[41,42] The mother's cortisol level is expected to increase at least two-fold throughout pregnancy.[43] During this time the fetus is exposed to increasing concentrations of cortisol.

One hypothesized pathway for how maternal stress affects the fetus's neurodevelopmental outcome is through the HPA axis. While the fetal hypothalamus begins forming around 9-10 weeks of gestation, it is not fully able to function until early in the second trimester of pregnancy.[44] It is theorized that ASD can develop because the fetus begins to experience the mother's stress during the second trimester. High stress causes the fetus's HPA axis function to be altered to a higher set point or greater reactivity, which in turn suppresses the fetus's immune response. During the gestational period when the blood-brain barrier (BBB) of the fetus is not fully developed, antibodies and other larger molecules have greater access to the brain.[45] Trauma or stress increases BBB permeability which enhances the risk of exposing the brain to environmental stimuli and insults that could impact neurodevelopment,[46] resulting in atypical development, such as ASD.[47]

Cortisol is the body's main stress hormone and is the most common way to measure stress biologically. Measurement of cortisol prenatally has been widely assessed in rodent and non-human primate animal models and found to be associated with autism-like behaviors[7-10]. Prenatal cortisol has been less commonly studied experimentally in humans due to the ethical concerns of inducing prenatal stress and there have been few prospective studies to investigate the association of prenatal cortisol and neurodevelopmental outcomes.

The common methods for measuring cortisol have primarily been through salivary or serum samples. However, these sample collections are practically challenging to collect due to the nature of cortisol secretion being episodic and exhibiting a circadian rhythm.[48] Cortisol concentrations in the human body are at their highest in the morning and decline throughout the day. To accurately measure cortisol and

compare across participants would require samples to be collected at the exact same time with repeat collections throughout the day. This approach was not feasible with this cohort due to staffing availability and burden on pregnant women and their families. Using 24-hr urine samples for cortisol is an established clinical collection method used for screening in Cushing's syndrome in which there is an overproduction of cortisol, called hypercortisolism.[49] Cortisol production rate is difficult to measure directly so the best method for diagnosis of hypercortisolism is through daily urine free cortisol excretion, measured in a 24-hr urine collection where creatinine is also determined to evaluate the completeness of the urine collection. There is an upper limit of normal daily urine free cortisol excretion. Laboratories take into account pregnancy when determining 24-hr urine collection completeness, in which the upper limit of daily cortisol production rate and urine free cortisol excretion are elevated.

Because detecting cortisol production for Cushing's, a disease detecting overproduction of cortisol, is an accurate and trusted method, laboratories comfortably use this 24-hr urinary assay for measuring cortisol when the gold standard salivary cortisol is not feasible or available[48]. This study is, to the authors' knowledge, the first to use 24-hr urine samples during pregnancy to measure cortisol in association with child ASD and Non-TD neurodevelopmental outcomes.

Previous studies have found diurnal cortisol rhythms to differ based on racial and ethnic background, with Black and Hispanic groups having more subtle declines throughout the day compared to whites.[50,51] This study examined if race interacts with cortisol in the association between cortisol and neurodevelopmental outcome. The associations between prenatal cortisol and ASD were hypothesized to be stronger in non-white mother-child pairs than white mother-child pairs, due to cumulative effects of known cortisol pattern differences and other (unmeasured) stressors related to racism and discrimination. Additionally, due to the unequal ASD prevalence in males and females (4:1)[52] and biological plausibility of sex difference in ASD and Non-TD etiology,[53,54] this study also investigated if

there was an interaction between sex and cortisol in the association between cortisol and neurodevelopmental outcome.

To date, the authors are aware of one study that has prospectively studied the association of fetal cortisol exposure and ASD symptoms at age 5.[33] The study found that fetuses exposed to lower levels of maternal cortisol were associated with more ASD symptoms in boys. The study used the Social Communication Questionnaire to gauge ASD symptoms. This present study prospectively investigated diagnosed ASD as the outcome, where expert clinicians assessed and confirmed the ASD diagnosis of the child. This is also the first study to examine the association with recurrence in a high familial risk population. Siblings of children with ASD are at elevated risk (~20%) of also having ASD, compared to the general population (~1.5%).[27] With a growing number of families having a child diagnosed with ASD, the need for research specific to these families becomes even more important. This study will provide insight into prenatal risk factors for these high familial risk families and help inform their decisions when growing their families.

OBJECTIVE/HYPOTHESIS

The main objective of this study is to examine the association between prenatal urinary cortisol and child neurodevelopmental outcomes. A subobjective is to examine this association stratified by race. It is hypothesized that cortisol will be significantly associated with ASD and Non-TD outcomes and that this association differs by race, with the association being stronger in non-white races. This could be because non-white mothers' perceived stress is compounded by structural racism, disparities in health care, microaggressions, stressors of assimilating, and lack of access to culturally sensitive support.

METHODS

Study population

This study included 146 pregnant mothers that participated in the Markers of Autism Risk in Babies: Learning Early Signs (MARBLES) study[67]. After birth, these children also participated in MARBLES through 3 years of age. MARBLES is a longitudinal cohort study that enrolled high familial risk pregnant women. Selection criteria to be enrolled in the study were: 1) have at least one biological child diagnosed with autism or ASD and so have greater likelihood of having another child with ASD;[27] 2) be pregnant (or carrying the child of a male that is a biological father of a child with ASD) or planning a pregnancy; 3) live within a 2hr radius of the Davis/Sacramento area; 4) be at least 18 years old; and 5) be able to speak read, and understand English and their child will be raised with English as one of their primary languages.

The majority of eligible families were identified through the California Department of Developmental Services (DDS), which provided the study with a list of families that have received or are receiving state-funded services for a child with ASD. Other families were also enrolled through outreach events, word-of-mouth, referred by other studies at the UC Davis MIND Institute, or referred by other health providers.

The diagnosis of the older sibling with ASD was confirmed in order to be enrolled in the study. Study psychologists reviewed records of evaluations for the sibling to confirm ADOS scores. If the psychologists were unable to obtain these records or scores are not present, an ADOS was administered in-house at the MIND Institute to confirm that the child met ASD criteria.

For the current study, 146 mother-child pairs were included because: 1) they provided a 24-hr urine collection sample in the first trimester and may also have provided 24-hr urine collections in the second

and/or third trimesters, and 2) their child later completed the study at 3 years of age and had a confirmed neurodevelopmental outcome.

Cortisol assessment

Mothers provided a 24-hr urine sample once every trimester during pregnancy. Depending on trimester at enrollment, each mother provided up to a total of three 24-hr urine collections, where all urine output for a 24-hr period was collected.

The 24-hr urine samples collected from participants' in-home visits were taken to the UC Davis Department of Public Health Sciences biorepository where a sample of the aggregate was then taken for assay and indexed by volume of output for the 24-hr period. Aliquots were shipped to the Endocrine Core Lab with the UC Davis Center for Health and the Environment, where the assays were completed. The AVIDA Centaur CP two site chemiluminescent immunoassay for cortisol involves competitive binding of cortisol in unknown samples with acridinium ester-labeled cortisol to a polyclonal rabbit anti-cortisol antibody in the solid phase. The polyclonal anti-cortisol antibody is bound to monoclonal mouse anti-rabbit antibody covalently coupled to paramagnetic particles for separation. Acid and base reagents initiate the chemiluminescent reaction and the intensity of the reaction is measured in relative light units (RLUs). An inverse relationship exists between the amount of cortisol present in the unknowns and the relative light units detected by system. Urinary creatinine was measured by colorimetric protein assay utilizing the Jaffe reaction.[96]

Urinary cortisol deviations

Urinary cortisol levels for each trimester were first analyzed to examine whether baseline cortisol levels were associated with neurodevelopmental outcome. Then, urinary cortisol levels for each trimester were analyzed by creating new variables indicating the deviations of the trimester levels from the average cortisol level throughout pregnancy for each mother. Creating these deviation variables allowed

for consideration that some mothers in the study may have higher baseline cortisol levels than other mothers. Without examining deviations, analyses would assume that the differences between mothers was the same as the differences within each individual mother. However, recognizing that there may be differences within each mother as well, using the deviation variables with the total pregnancy averages allowed for consideration of these differences.

Child neurodevelopmental assessment

Child neurodevelopment was assessed longitudinally from birth through three years of age by UC Davis MIND Institute licensed clinical psychologists who administered the gold standard Autism Diagnostic Observation Schedule (ADOS), a semi-structured interview during which the clinician observes social interaction, communication, play, and imaginative use of materials.[68] The clinician also administered the Mullen Scales of Early Learning (MSEL), using subscales that measure fine motor, visual reception, and expressive and receptive language.[69] An algorithm, previously published by the Baby Siblings Research Consortium,[27] was used to classify children into one of the following groups: typically developing (TD), ASD, or non-typically developing (non-TD). Children with ASD outcomes (*n*= 36) had scores over the ADOS cutoff and met the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for ASD. Children with non-TD outcomes (*n*=21) had scores within three points of the ADOS cutoff and/or MSEL scores 1.5 to 2 standard deviants below average.[27] The remaining children were classified as TD (*n*=89). Child outcome at 36-months was used in this present study.

Confounding Assessment

In addition to the cortisol measure in each trimester, other covariates in the final model were selected based on a Directed Acyclic Graph (DAG) presented in **Figure 1** that was created *a priori*. The figure shows arrows signifying known associations from exiting literature, with bold red arrows showing known associations specifically from literature in high familial risk populations. The primary model of this study

has the advantage of adjusting for a sufficient set of confounders based on subject matter expertise in high familial risk populations. All backdoor paths were identified and the minimally sufficient set to block these paths were: maternal race/ethnicity, maternal age, and home ownership as a proxy for socioeconomic status. Number of thaws for each sample and creatinine level were *a priori* determined to be in the final model to account for variations in urine samples and so these two covariates were added to the minimally sufficient set determined from the DAG.

Because there are more widely known associations with ASD in general population studies, a sensitivity analysis was conducted to consider additional potential confounding variables. These associations with outcome are shown with blue arrows in **Figure 1**. The maternal psychiatric conditions variable was not included in analyses due to unavailability of these data. Otherwise, the covariates for the minimally sufficient set in the sensitivity analysis were: maternal race/ethnicity, maternal age, home ownership, immune stressors during pregnancy, maternal country of birth (Inside or Outside the US), parity, and environmental stressors (cotinine, carbon monoxide, nitrogen oxide, nitrogen dioxide, ozone – 24hr, adjusted PM10, PM2.5, monoethyl phthalate, bisphenol A, and ethyl paraben).

Race and ethnicity were collected from participants through multiple sources, which included the Environmental Exposure Questionnaire phone interview, the Family Information Form, the MARBLES database tracking system, and California birth files. Responses from four sources were self-reported by the participant. To determine race, participants were asked if they identified as: white, Black/African American, American Indian/Alaska Native, Asian, Native Hawaiian/Other Pacific Islander, Other, or more than one race. To determine ethnicity, participants were asked if they identified as Hispanic or not Hispanic. Because this information was collected multiple times, if responses did not align, MARBLES programmers manually reviewed participants to determine any errors and correctly classified race/ethnicity. Due to small sample size and low frequencies in the African American, Hispanic,

American Indian, and mixed race groups, race and ethnicity were collapsed into three categories: non-Hispanic white and Other.

Home ownership information was used as an indirect measure for SES and was collected through the MARBLES Environmental Exposure Questionnaire (EEQ).

Immune stressors were comprised of any infections moms may have had during pregnancy. This information was collected through the EEQ. Mothers reported, if any, which months they had the following infections: chicken pox, flu, measles, mumps, rubella, shingles, gonorrhea, syphilis, toxoplasmosis, cytomegalovirus, hepatitis, genital herpes, urinary tract infection, pelvic inflammatory disease (PID), chlamydia, trichomonas, bacterial vaginosis, vulvoginal yeast, sinusitis, bronchitis, tuberculosis, Lyme's disease, other vaginal infections (asked to specify), other respiratory infections besides a cold (asked to specify type), and any other infections (asked to specify type). Because there was not enough variability for a robust estimation for each trimester, an immune risk score was calculated for each participant to reflect any infections throughout all of pregnancy. Scores were collapsed into a dichotomous score of "0" or "1 or more" due to low frequencies. Even with this collapse, there was not enough variability for estimation so the variable was dropped from the sensitivity analysis.

Maternal country of birth was collected through CA Birth Files of the child. If not stated in the birth files, the EEQ phone interview was used, where mothers reported their place of birth. Responses were coded to indicate if mothers were born inside or outside of the United States.

Parity was collected through the EEQ. Any pregnancies over 20 weeks gestation prior to the child of interest's birth were considered, no matter if the pregnancy resulted in miscarriage, abortion, still at

birth, or live birth. Categories were collapsed to “1” or “2+” due to allow variability for a more robust estimation.

Maternal age at delivery was calculated using maternal date of birth and child’s date of birth.

Environmental exposures were comprised of smoking information (cotinine), air pollution, and chemical exposures. Cotinine was calculated from prenatal maternal urine samples with the limit of detection being 0.2 ng/mL. However, cotinine was measured in only a small subset of women so the variable was dropped due from the sensitivity analysis to a large amount of missing data.

Air pollution information, namely ambient CO, NO, NO2, O3, PM2.5 and adjusted PM10 air pollution monthly exposure, were calculated based on residential history collected for all participants. The pregnancy exposures were estimated from data downloaded from the U.S. Environmental Protection Agency’s (EPA) Air Quality System (AQS) database [87] that provides daily average air pollution concentrations which are spatially interpolated to residential addresses for each participant. Monthly averages were calculated from the daily averages, and these daily averages were spatially interpolated from the air quality monitoring stations’ locations to the residence locations using inverse distance-squared weighting. An adjusted estimate for PM10 was equal to PM2.5 when the originally estimated PM10 was less than the corresponding PM2.5 and the distance to the nearest monitoring station was shorter for the PM2.5 estimate than the original PM10 estimate.

Chemical exposures were selected to test for confounding based on findings from previous MARBLES investigations of environmental exposures[88,89]. Chemicals examined for possible confounding were: the phthalate (metabolite) MEP,[88] the phenol BPA,[89] and the paraben EtPB.[89] Methods for determining exposure based on concentrations in urine have been previously published.[88,89] In short, up to four urine samples were collected from each mother in the second and third trimesters of pregnancy. Specific

gravity-corrected averages for each trimester were calculated and used for confounding assessment. Because MEP, BPA, and EtPB were measured in only a small subset of participants in this study, these variables were dropped from the sensitivity analysis due to the large amount of missing data.

Statistical analyses

Exposure of Interest – Cortisol

Cortisol measures for each trimester were checked for normality and outliers. Creatinine was used to evaluate hormone values in the urinary assays, as urinary levels can vary with hydration. Samples where creatinine values were <0.20 mg/mL were excluded, as these outliers were an indication that the urine sample was likely too dilute to yield reliable measurements, and if kept in the analysis, could result in falsely elevated cortisol levels. The cutoff of 0.20 mg/mL is standard for urinary samples, even when considering this pregnancy cohort where creatinine levels are expected to decrease.[97] After excluding these outliers, the trimesters' cortisol levels were natural log transformed to better approximate a normal distribution. To present characteristics of the study population by exposure (**Table 1**), cortisol averages for each individual were calculated. The median cortisol concentration of the total population was calculated and used to categorize exposure as less than or equal to or greater than the median value. Dichotomized median split scores for cortisol concentrations were used solely to present information in **Table 1**; continuous cortisol concentrations were used as the exposure of interest in all other analyses.

Multinomial Logistic Regressions

To examine the association of prenatal cortisol and neurodevelopmental outcome, three multinomial logistic regressions were fitted for ASD and Non-TD outcome, controlling for maternal race/ethnicity, maternal age, home ownership, number of thaws in the sample, and creatinine level, with the exposure of interest being the respective natural log transformation of the cortisol variable. In the third trimester,

non-TD outcome was not examined due to small sample size (n=5) and missing covariate data for these five participants. A multinomial logistic regression was also fitted for the entire pregnancy, where cortisol was averaged across all trimesters.

With the ratio of ASD in males to females being unequal (4:1)[52] and there being biologic plausibility for sex differences in ASD and Non-TD etiology,[54,71] interaction between cortisol and child sex was examined in the association between cortisol and child neurodevelopmental outcome.

Multinomial logistic regression models were fitted, also controlling for the six covariates listed above, to examine the relationship of prenatal cortisol deviations in each trimester and neurodevelopmental outcome. Again, non-TD outcome in the third trimester could not be examined due to small sample size. A multinomial logistic regression was also fitted for the entire pregnancy, where cortisol deviations were averaged across all trimesters.

Multinomial logistic regressions were then fitted for natural log transformed cortisol variables of each trimester with neurodevelopmental outcome, controlling for maternal age, home ownership, pregnancy NO_2 average, number of thaws in the sample, and creatinine level, including an interaction term with cortisol and race to test if sex-differences were present. Due to low frequencies, race was collapsed into a dichotomous variable of either non-Hispanic white or Other. Cortisol and Non-TD outcome interactions were not examined in trimester 3 due to small frequencies.

All multiple logistic regressions from the above primary analysis were fitted for the sensitivity analysis, which considered additional potential confounding variables.

Receiver Operating Characteristic Curves

Perceived stress was previously investigated as the stress exposure of interest. In this study, receiver operating characteristic (ROC) curves were graphed for perceived stress scale total average and for

cortisol total average separately using the primary analyses in order to compare predictability of ASD outcome of the two stress measurement methods. In the ROC curve for perceived stress, in addition to the exposure of interest, covariates included were: maternal race/ethnicity, maternal age, and home ownership. In the ROC curve for cortisol, other covariates included were: maternal race/ethnicity, maternal age, home ownership, number of thaws in the sample, and creatinine level. To test if the ASD predictability for each stress method was meaningfully different from one another, a paired ROC curve was graphed with perceived stress and cortisol in a combined model. Covariates from each independent model were included in the combined model.

Statistical analyses were performed with SAS version 9.4 (Institute Inc. Carny, NC, USA). Results with p-values≤ 0.05 were considered statistically significant.

RESULTS

A total of 146 pregnant mothers were included in this study. The majority of mothers were non-Hispanic white, followed by Asian, Hispanic, Mixed Race/Other, and then African American/Black. The proportions of race and ethnicity in the total study sample is also shown by each outcome group of ASD, non-TD, and TD. The majority of mothers were homeowners and born in the U.S. Most mothers did not experience immune stressors, such as infections, during pregnancy. The distribution of participants across seasons of conception was fairly equal. The mean age for mothers was 34.8 years in the total study population. The mean age in the TD and ASD groups were 35.04 years, and the mean age in non-TD was 33.6 years. More details on the characteristics of study participants stratified by child neurodevelopmental outcome can be found in **Table 1**. Characteristics of study participants stratified by median cortisol level of 17.80 ug/dL are presented in **Table 2**.

All results exploring regressions with cortisol levels, cortisol deviations, and cortisol stratified by sex were non-significant, indicating that cortisol levels during pregnancy are not associated with neurodevelopmental outcome, nor do these results differ meaningfully by white and Other race (**Tables 3-6**). However, it is worth mentioning that relative risk ratios were generally all below 1.0, indicating a possible protective effect with increased cortisol. This finding warrants further investigation, as will be discussed in the next section. When examining race-specific relative risk ratios of cortisol and ASD outcome, non-Hispanic white race results were statistically significant (RRR=0.16, 95% CI (0.03, 0.97)). The natural log transformation of cortisol can be exponentiated for easier interpretation. With a beta value of -1.8493 for the natural log of cortisol, this means that a 1% increase in cortisol is associated with a decrease of approximately 0.18% in ASD:TD risk ratio. The sample size for this analysis was relatively small (n=27) so further research is necessary.

Two ROC curves were created to examine if perceived stress or cortisol would be a better predictor of ASD outcome. The area under the curve (AUC) for perceived stress was 0.66 (95%CI (0.58, 0.74)) (**Figure 2, Table 7**), while the AUC for cortisol was 0.54 (95%CI (0.43, 0.68)), seen in **Figure 3 and Table 7**. Both measures of stress were fitted into one model to graph paired ROC curves in order to compare the two curves statistically. AUCs for each stress measure did not change substantially when fitted into one model (**Figures 2-4**). Comparing these two curves did not show they were meaningfully different (p-value 0.14) (**Table 7**).

Adding additional potential covariates to the model for the sensitivity analysis did not change estimates substantially (**Tables 8-11**), and all associations were in the same direction as the primary analysis.

DISCUSSION

This study was the first to examine urinary cortisol as a biomarker for prenatal stress and its association with neurodevelopmental outcome in a high familial risk cohort.

The sample size for this study was relatively small, thus it is highly possible that smaller effects were unable to be detected, resulting in non-significant findings. In the second trimester, increased cortisol was associated with increased risk of ASD, though not statistically significant. In first trimester analyses, increased cortisol was associated with decreased risk of ASD and Non-TD, but again, non-significant with low precision. However, the general direction of findings in this study agree with another prospective study conducted which found an association between lower maternal cortisol and increased ASD symptoms in the child.[33] That investigation did not find a main effect association between cortisol and ASD symptoms, but an inverse association was present in males when including an interaction term between maternal cortisol and sex. The current study attempted to confirm these findings in the MARBLES cohort, but small sample sizes and frequencies by sex limited the ability to replicate results.

Decreased cortisol during pregnancy and elevated risk of ASD could be explained by mothers having a decreased reaction to stressors due to chronic stress associated with already caring for a child with ASD. This chronic stress would alter HPA-axis function[98] so her change in cortisol levels from additional stressors might be lower than expected. This explanation would be in agreement with existing studies that have found that mothers of children with ASD had decreased awakening cortisol levels compared to mothers of children without ASD.[99,100]

In regards to the analysis by race, which was dichotomized as non-Hispanic white or Other, it is again possible that smaller differences were not detected due to sample size. In addition, cortisol rhythms differ by race[101] so having a collapsed Other category that included individuals of several different races/ethnicities could have washed out any differences that may have been present between these

groups, especially since previous investigations have found diurnal cortisol patterns change less steeply among Black and Hispanic adults compared to white adults.[50,51] Enrolling more mothers of all races would allow for a more powerful examination of this association.

Comparing the two ROC curves of perceived stress and cortisol suggests that perceived stress may be a better predictor of ASD outcome. The prediction for perceived stress was statistically significantly better than chance alone, with the area under the curve being 0.66, which indicates that there is roughly a 66% chance that the model will be able to distinguish between ASD and TD outcomes. The AUC for cortisol was not statistically significant (AUC=0.55, 95% CI (0.43, 0.68)). However, this possible higher predictability from the perceived stress model compared to the cortisol model could be explained by the fact that mothers may perceive stress to take a bigger or smaller toll her on based on how much stress she is accustomed to feeling on a daily basis. For example, if a mother is not used to having stressful situations arise, she might perceive stressful events to be more impactful than a mother who experiences stressful situations regularly. Cortisol is a stress-related hormone, but its activity is impacted by factors other than stress. Thus, this biological response may not be as predictive since fluctuation of cortisol is downstream of how the mother perceives the stressor and may also be impacted by other factors, such as diet or sleep.

All estimates in the sensitivity analysis remained in the same direction as the primary analysis so there were no changes when fitting additional potential confounders. However, relative risk ratio changes in the sensitivity analysis were varying when considering if they resulted in higher or lower estimates than the primary model. This could be due to fitting variables that were missing data or excluding potential confounders due to large amounts of missing data. Measuring covariates for all participants in the study will result in more precise estimates.

In this present study, using a snapshot of cortisol at one point in pregnancy does not show significant associations with these outcomes. Compared to previous investigations which measured stress through stressful life events and did not find an association with neurodevelopmental outcome and perceived stress which was found to be associated with neurodevelopmental outcome, perhaps it is not the mother experiencing these stressful life events or how her body biologically responds to the events that impact the risk of the child, but instead how the mother perceives these events that she experiences. It is also possible that, because perceived stress scores were collected multiple times in each trimester, the perceived stress measure was more sensitive to changes, whereas 24-hr urine collections happened only once during each trimester so may not be representative of cortisol throughout a 13-week trimester.

As mentioned, one limitation of this study was the small sample size which limited our power to detect small effect sizes and to stratify results by sex and race. Future research enrolling more high familial risk mothers of various races are needed in order to fully examine the association of cortisol and ASD or non-TD risk. Another limitation of this study is that the 24-hr urinary sample was collected only once per trimester so may not be fully representative of the whole trimester's cortisol levels and the changes within that trimester. Multiple 24-hr urine collections would strengthen the precision of cortisol measurement for the trimester. There are multiple strengths to this study. To the authors' knowledge, this is the first study to use 24-hr urinary cortisol assays to study stress. Other studies have primarily used spot urine cortisol assays (collected at one time during the day) or serum cortisol, but some were not able to collect the samples for all mothers at the same time of the day. These studies were not able to account for biological changes in cortisol throughout the day. This study used a 24-hr sample, which collected all urine output during the day and thus acts as a way to take an "average" of the cortisol level fluctuations throughout the whole day, which is also less burdensome on study staff and participants to coordinate times of collection. Another strength of this study is that it was prospectively conducted.

Mothers provided urine sample during their pregnancy, and once their child was born, the child's neurodevelopment was assessed periodically through age 3. This allows for a stronger temporal relationship between exposure and outcome, as opposed to a retrospective study where recollection of exposures preceding the outcome might be more susceptible to biases.

This study did not find evidence for an association between prenatal cortisol and neurodevelopmental outcomes, but adds to the growing knowledge of prenatal stress exposure as a risk factor for ASD or Non-TD outcomes in the child. In addition to considering cortisol as a stress biomarker, future studies should also incorporate the assessment of stressful life events and perceived stress. Future research is also needed to understand these stress exposures and how they are correlated with each other and neurodevelopmental outcomes. A better understanding of these forms of stress will result in more opportunities to mitigate stress and create optimal environments for pregnant mothers to enhance the long-term neurodevelopmental outcomes of her child.

APPENDIX

Figure 1. Directed Acyclic Graph (DAG)

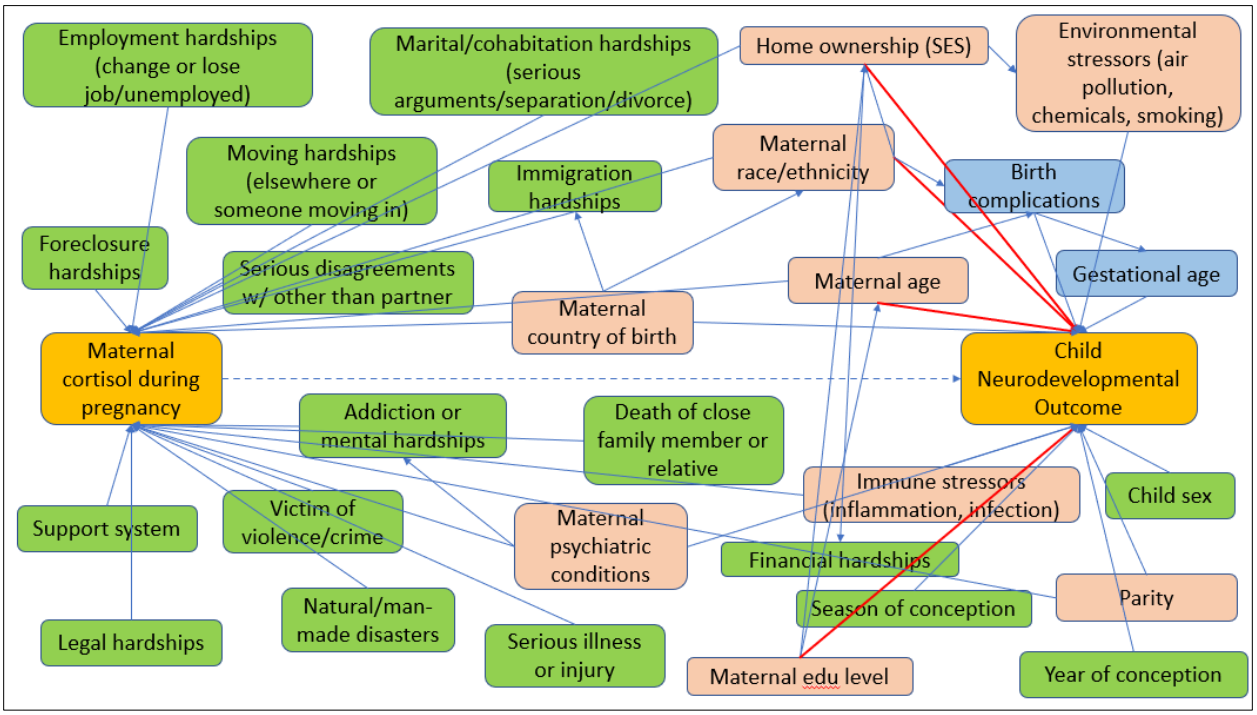

Figure 1. Directed Acyclic Graph created to select covariates to control for a priori. Exposure variable is maternal cortisol during pregnancy. Outcome is child neurodevelopmental outcome. Variables in green are upstream of the exposure variable. Variables in blue are on the pathway from exposure to outcome or are downstream of outcome. Variables in red are confounders. Blue arrows show associations of variables from existing literature in general population studies. Bold red arrows show associations of variables from existing literature in high familial risk populations. Variables selected as the minimally sufficient set to block all backdoor paths in the primary analysis were: maternal race/ethnicity, maternal age, and home ownership as a proxy for socioeconomic status (SES). Number of thaws for each sample and creatinine level were a priori determined to be in the final model to account for variations in urine samples and so these two covariates were added to the minimally sufficient set determined from the DAG. Variables selected as the minimally sufficient set in addition to the primary analysis variables were: maternal psychiatric conditions, immune stressors, maternal country of birth, parity, cotinine, ambient carbon monoxide, nitrogen oxide, nitrogen dioxide, ozone – 24hr, adjusted PM10, PM 2.5, monoethyl phthalate, Bisphenol A, and ethylparaben).

Figure 2. ROC Curve for Prenatal Perceived Stress Score

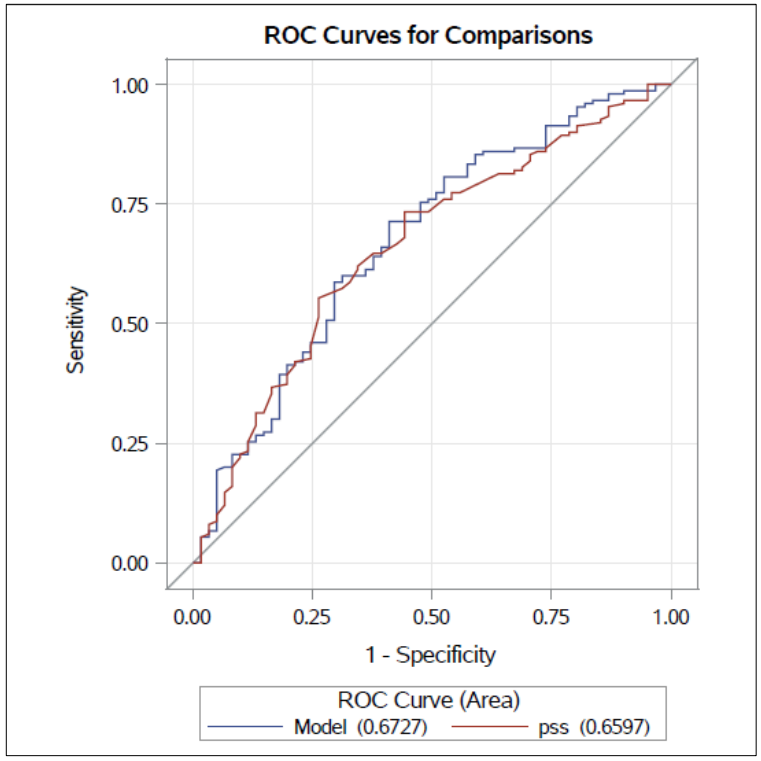

Figure 2. ROC Curve graphing prenatal perceived stress score as a predictor for ASD outcome in the child. Perceived stress score graphed was an average taken of all perceived stress scores during pregnancy. The model controlled for maternal race/ethnicity, maternal age, and home ownership.

Figure 3. ROC Curve for Prenatal Urinary Cortisol

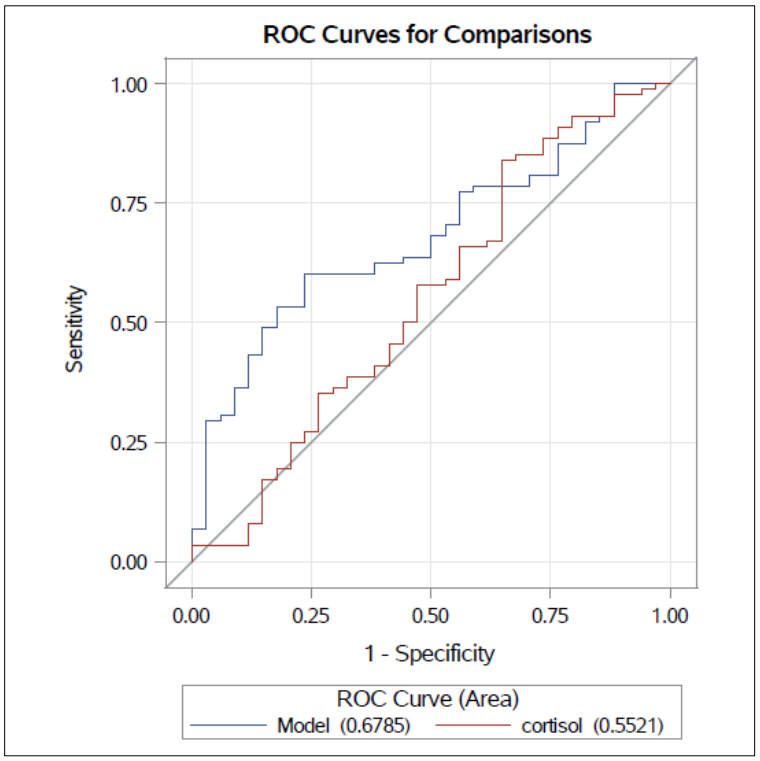

Figure 3. ROC Curve graphing prenatal urinary cortisol as a predictor for ASD outcome in the child. Cortisol graphed was an average of all cortisol measures during pregnancy. The model controlled for maternal race/ethnicity, maternal age, home ownership, creatinine, and number of thaws in the urine sample aliquot.

Figure 4. Paired ROC Curves for Prenatal Perceived Stress Score and Cortisol

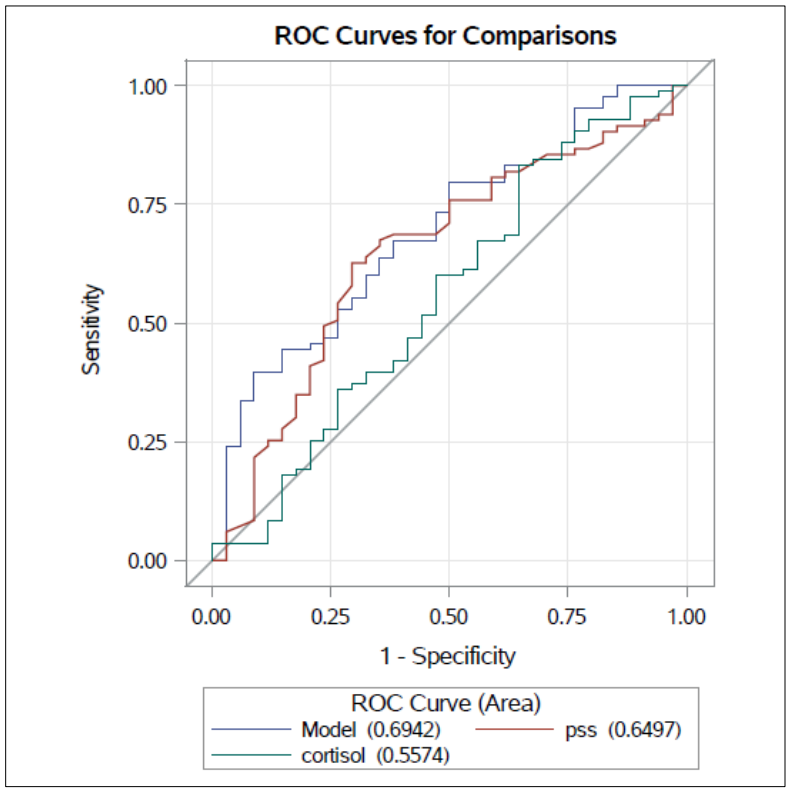

Figure 4. ROC Curves graphing prenatal urinary cortisol and cortisol as predictors for ASD outcome in the child. Perceived stress and cortisol graphed were averages of their respective measures throughout pregnancy. The model controlled for maternal race/ethnicity, maternal age, home ownership, creatinine, and number of thaws in the urine sample aliquot. The AUCs here did not change substantially when fitting one combined model compared to the two separate models in Figures 2 and 3.

Table 1. Characteristics of study participants, stratified by child neurodevelopmental outcome

Characteristics	Row total		TD		ASD		Non-TD		p-value
	n	%	n	%	n	%	n	%	
Mother Race/Ethnicity									0.31
Non-Hispanic White	78	53.42	45	50.56	21	58.33	12	57.14	
Hispanic	21	14.38	15	16.85	4	11.11	2	9.52	
Black/African American	4	2.74	0	0	2	5.56	2	9.52	
Asian	31	21.23	20	22.47	6	16.67	5	23.81	
Mixed Race/Other	12	8.22	9	10.11	3	8.33	0	0	
Homeowner									0.2
No	57	39.86	31	35.23	18	52.94	8	38.1	
Yes	86	60.14	57	64.77	16	47.06	13	61.9	
Immune Stressors									
Trimester 1									0.72
0	145	99.32	88	98.88	36	100	21	100	
1+	1	0.68	1	1.12	0	0	0	0	
Trimester 2									0.35
0	139	95.21	83	93.26	35	97.22	21	100	
1+	7	4.79	6	6.74	1	2.78	0	0	
Trimester 3									
0	127	86.99	77	86.52	30	83.33	20	95.24	
1+	19	13.01	12	13.48	6	16.67	1	4.76	
Maternal Country of Birth									0.95
Inside the U.S.	100	69.44	62	69.66	24	70.59	14	66.67	
Outside the U.S.	44	30.56	27	30.34	10	29.41	7	33.33	
Season of Conception									0.87
Fall	46	31.51	26	29.21	13	36.11	7	33.33	
Winter	31	21.23	18	20.22	7	19.44	6	28.57	
Spring	32	21.92	20	22.47	7	19.44	5	23.81	
Summer	37	25.34	25	28.09	9	25	3	14.29	

Parity												0.998
1	67	72.04		44	72.13		13	72.22		10	71.42	
2+	26	27.96		17	27.87		5	27.78		4	28.57	
1st trimester 24hr-urine aliquot - number of thaws												0.72
0	80	54.79		52	58.43		18	50		10	47.62	
1	65	44.52		36	40.45		18	50		11	52.38	
2	1	0.68		1	1.12		0	0		0	0	
2nd trimester 24hr-urine aliquot - number of thaws												0.44
0	57	45.6		38	48.72		14	45.16		5	31.25	
1	68	54.4		40	51.28		17	54.84		11	68.75	
3rd trimester 24hr-urine aliquot - number of thaws												0.77
0	24	48.98		16	50		5	41.67		3	60	
1	25	51.02		16	50		7	58.33		2	40	
	Total			**TD**			**ASD**			**Non-TD**		**p-value**
	n	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	
Maternal Age (years)	146	34.84	4.39	89	35.04	4.46	36	35.04	4.49	21	33.64	3.85	0.38
Cotinine (ng/mL)	118	29.3	164.77	70	15.65	106.6	29	81.18	285.67	19	0.43	0.38	0.58
Carbon Monoxide (ppb)	114	0.34	0.08	67	0.34	0.08	29	0.34	0.08	18	0.35	0.09	0.4
Nitrogen Oxide (ppb)	114	6.58	3.43	67	6.59	3.37	29	6.77	3.74	18	6.24	3.32	0.74
Nitrogen Dioxide (ppb)	114	10.1	2.56	67	10.17	2.56	29	10.23	2.83	18	9.82	2.21	0.21
Ozone - 24hr (ppb)	115	25.13	4.06	68	25.16	4.02	29	24.41	3.89	18	26.22	4.47	0.43
Adjusted PM10 (ug/m3)	114	19.69	4.36	67	20.04	4.58	29	18.21	3.79	18	20.76	3.96	0.08
PM2.5 (ug/m3)	115	9.9	2.3	68	10.13	2.39	29	9.05	1.92	18	10.42	2.29	0.4
Monoethyl phthalate (MEP) (µg/L)													
Trimester 2	76	43.6	48.53	45	38.41	51.55	17	48.18	41.78	14	54.72	46.82	0.74
Trimester 3	76	81.8	366.26	46	33.81	30.28	17	58.71	63.7	13	281.8	881.28	0.27
Bisphenol A (BPA) (ng/mL)													
Trimester 2	84	1.64	1.24	49	1.6	1.19	20	1.47	1.11	15	1.96	1.54	0.99
Trimester 3	82	2.07	2.92	47	2.17	3.58	21	1.94	1.64	14	1.95	1.87	0.66
Ethyl paraben (EtPB) (ng/mL)													
Trimester 2	84	14.16	62.35	49	9.25	18.65	20	34.5	124.51	15	3.08	3.19	0.9

Trimester 3	82	7.99	15.33	47	8.14	17.06	21	7.64	11.94	14	7.99	14.65	0.85
Creatinine (mg/mL)													
Trimester 1	146	0.92	0.5	89	0.83	0.41	36	1.07	0.61	21	1.06	0.59	0.02
Trimester 2	125	0.84	0.44	78	0.76	0.37	31	0.94	0.37	16	1.07	0.7	0.02
Trimester 3	49	0.69	0.33	32	0.75	0.33	12	0.62	0.33	5	0.48	0.2	0.19

Table 1. Characteristics of MARBLES mothers included in the analyses by neurodevelopmental outcome group. Immune stressors were determined from calculating a risk score based on how may infections mothers reported during each trimester of pregnancy. Infections recorded are listed in the methods section. P-values were obtained from chi square tests. Note: TD=typically developing, non-TD=nontypically developing, ASD=autism spectrum disorder.

Table 2. Characteristics of study participants, stratified by cortisol level

Characteristics	Row total		Individual cortisol average ≤ total median cortisol (17.80 ug/dL)		Individual cortisol average > total median cortisol (17.80 ug/dL)		p-value
	n	%	n	%	n	%	
Mother Race/Ethnicity							0.002
Non-Hispanic White	78	53.42	40	54.79	38	52.05	
Hispanic	21	14.38	6	8.22	15	20.55	
Black/African American	4	2.74	0	0	4	2.74	
Asian	31	21.23	23	31.51	8	10.96	
Mixed Race/Other	12	8.22	4	5.48	8	10.96	
Homeowner							0.21
No	57	39.86	25	34.72	32	45.07	
Yes	86	60.14	47	65.28	39	54.93	
Immune Stressors							
Trimester 1							0.32
0	145	99.32	72	98.63	73	100	
1+	1	0.68	1	1.37	0	0	
Trimester 2							0.7
0	139	95.21	69	94.52	70	95.89	
1+	7	4.79	4	5.48	3	4.11	
Trimester 3							0.81
0	127	86.99	63	86.3	64	87.67	
1+	19	13.01	10	13.7	9	12.33	
Maternal Country of Birth							0.04
Inside the U.S.	100	69.44	45	61.64	55	77.46	
Outside the U.S.	44	30.56	28	38.36	16	22.54	
Season of Conception							0.99
Fall	46	31.51	24	32.88	22	30.14	
Winter	31	21.23	15	20.55	16	21.92	
Spring	32	21.92	16	21.92	16	21.92	
Summer	37	25.34	18	24.66	19	26.03	

Parity										0.26
1	67	72.04		37	77.08		30	66.67		
2+	26	27.96		11	22.92		15	33.33		
1st trimester 24hr-urine aliquot - number of thaws										0.4
0	80	54.79		43	58.9		37	50.68		
1	65	44.52		30	41.1		35	47.95		
2	1	0.68		0	0		1	1.37		
2nd trimester 24hr-urine aliquot - number of thaws										0.82
0	57	45.6		28	46.67		29	44.62		
1	68	54.4		32	53.33		36	55.38		
3rd trimester 24hr-urine aliquot - number of thaws										0.48
0	24	48.98		14	45.16		10	55.56		
1	25	51.02		17	54.84		8	44.44		
	Total			**Individual cortisol average ≤ total median cortisol (17.80 ug/dL)**			**Individual cortisol average > total median cortisol (17.80 ug/dL)**			**p-value**
	n	**mean**	**SD**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	
Maternal Age (years)	146	34.84	4.39	73	35.85	3.52	73	33.82	4.93	0.005
Cotinine (ng/mL)	118	29.3	164.77	60	40.89	206.88	58	17.32	105.52	0.44
Carbon Monoxide (ppb)	114	0.34	0.08	56	0.35	0.09	58	0.33	0.08	0.19
Nitrogen Oxide (ppb)	114	6.58	3.43	56	6.93	3.77	58	6.25	3.07	0.29
Nitrogen Dioxide (ppb)	114	10.1	2.56	56	10.36	2.64	58	9.84	2.47	0.28
Ozone - 24hr (ppb)	115	25.13	4.06	57	24.95	4.22	58	25.32	3.93	0.62
Adjusted PM10 (ug/m3)	114	19.69	4.36	56	19.68	4.57	58	19.7	4.18	0.98
PM2.5 (ug/m3)	115	9.9	2.3	57	9.89	2.42	58	9.91	2.01	0.95
Monoethyl phthalate (MEP) (µg/L)										
Trimester 2	76	43.6	48.53	37	44.58	50.09	39	42.67	47.65	0.86
Trimester 3	76	81.8	366.26	37	35.7	32.15	39	125.54	509.66	0.29
Bisphenol A (BPA) (ng/mL)										
Trimester 2	84	1.64	1.24	43	1.69	1.45	41	1.58	0.99	0.7
Trimester 3	82	2.07	2.92	42	2.33	3.77	40	1.8	1.62	0.41

Ethylparaben (EtPB) (ng/mL)										
Trimester 2	84	14.16	62.35	43	20.5	86.28	41	7.51	12.76	0.34
Trimester 3	82	7.99	15.33	42	8.26	17.71	40	7.7	12.57	0.87
Creatinine (mg/mL)										
Trimester 1	146	0.92	0.5	73	0.64	0.24	73	1.21	0.53	0.001
Trimester 2	125	0.84	0.44	60	0.57	0.21	65	1.09	0.44	0.001
Trimester 3	49	0.69	0.33	31	0.55	0.26	18	0.92	0.3	0.001

Table 2. Characteristics of MARBLES mothers included in the analyses by individual cortisol average of either below or above the total median cortisol level of 17.80 ug/dL. Immune stressors were determined from calculating a risk score based on how may infections mothers reported during each trimester of pregnancy. Infections recorded are listed in the methods section. P-values were obtained from chi square tests. Note: TD=typically developing, non-TD=nontypically developing, ASD=autism spectrum disorder.

Table 3. Results of trimester natural log transformation of cortisol

Results of trimester natural log transformation of cortisol						
	ASD (vs. TD)			Non-TD (vs. TD)		
Time Point (total n)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value
Trimester 1 (n=143)	34	0.52 (0.12, 2.25)	0.38	21	0.52 (0.10, 2.71)	0.43
Trimester 2 (n=123)	30	1.32 (0.32, 5.41)	0.7	16	0.52 (0.08, 3.26)	0.48
Trimester 3 (n=48)	12	0.26 (0.03, 2.44)	0.24	5		
Pregnancy Average (n=143)	34	0.46 (0.10, 2.22)	0.34	21	0.29 (0.05, 1.75)	0.18

Table 3. Results from multinomial logistic regressions of cortisol scores in each trimester, adjusted for covariates. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and non-TD groups, with TD as the reference group. The Pregnancy Average timepoint reflects across all three trimesters. Relative Risk Ratio for non-TD in the third trimester is not presented due to small sample size.

Table 4. Results of sex-specific estimates of neurodevelopmental outcome for ln cortisol in ASD vs. TD

Female							
	ASD (vs. TD)			Non-TD (vs. TD)			
Trimester (n=total)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	Interaction p-value
Trimester 1 (n=62)	9	0.31 (0.08, 1.46)	0.14	10	0.53 (0.10, 2.90)	0.47	
Trimester 2 (n=52)	7	0.93 (0.21, 4.15)	0.92	7	0.52 (0.08, 3.28)	0.49	REF
Trimester 3 (n=23)	3			3			
Male							
	ASD (vs. TD)			Non-TD (vs. TD)			
Trimester (n=total)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	Interaction p-value
Trimester 1 (n=84)	27	0.46 (0.11, 1.99)	0.3	11	0.56 (0.11, 2.92)	0.49	0.08
Trimester 2 (n=73)	24	1.37 (0.32, 5.88)	0.67	9	0.59 (0.10, 3.66)	0.57	0.07
Trimester 3 (n=26)	9			2			

Table 4. Results from adjusted multinomial logistic regressions of ln cortisol in each trimester, adjusted for covariates to compare ASD vs. TD with an interaction term for ln cortisol and child sex. Total n's, sex-specific relative risk ratios, 95% confidence intervals, and p-values are presented. Associations in trimester 3 were not examined due to small cell sizes.

Table 5. Results of trimester natural log transformation of cortisol deviations

Results of trimester natural log transformation of cortisol deviations						
	ASD (vs. TD)			Non-TD (vs. TD)		
Time Point (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**
Trimester 1 (n=143)	34	1.01 (0.61, 1.67)	0.97	21	1.03 (0.57, 1.86)	0.93
Trimester 2 (n=123)	30	0.87 (0.46, 1.65)	0.67	16	1.06 (0.44, 2.52)	0.9
Trimester 3 (n=48)	12	0.37 (0.06, 2.40)	0.29	5		
Pregnancy Average (n=143)	34	0.96 (0.56, 1.62)	0.87	21	0.90 (0.48, 1.68)	0.73

Table 5. Results from multinomial logistic regressions of cortisol in each trimester, adjusted for covariates. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and non-TD groups, with TD as the reference group. The Pregnancy Average timepoint reflects across all three trimesters. Relative Risk Ratio for non-TD in the third trimester is not presented due to small sample size.

Table 6. Results of race-specific estimates of neurodevelopmental outcome for ln cortisol

non-Hispanic white							
	ASD vs. TD			Non-TD vs. TD			interaction p-value
Trimester (n=total)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	
Trimester 1 (n=78)	21	0.37 (0.07, 1.88)	0.23	12	0.32 (0.05, 1.97)	0.22	
Trimester 2 (n=65)	19	1.46 (0.35, 6.02)	0.6	9	0.54 (0.08, 3.54)	0.52	REF
Trimester 3 (n=22)	7	0.20 (0.04, 1.08)	0.06	1			
Other							
	ASD vs. TD			Non-TD vs. TD			interaction p-value
Trimester (n=total)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	
Trimester 1 (n=68)	15	0.82 (0.13, 5.07)	0.83	9	1.06 (0.13, 8.80)	0.96	0.43
Trimester 2 (n=60)	12	1.22 (0.30, 5.02)	0.78	7	0.52 (0.08, 3.21)	0.48	0.49
Trimester 3 (n=27)	5	0.16 (0.03, 0.97)	0.05	4			0.44

Table 6. Results from adjusted multinomial logistic regressions of ln cortisol in each trimester, adjusted for covariates to compare ASD vs. TD and Non-TD vs. TD. Total n's, race-specific relative risk ratios, 95% confidence intervals, and p-values are presented. RRRs were calculated from one model, which included an interaction term for PSS score and maternal race. Associations for Non-TD vs. TD in trimester 3 were not examined due to small cells sizes.

Table 7. Receiver operating characteristic (ROC) Curve Statistics

ROC Curve Association Statistics - ASD vs. TD			
Stress Measure	**Area Under the Curve (AUC)**	**95% CI**	**comparison of measures p-value**
Perceived Stress (pss)	0.66	(0.58, 0.74)	0.14
Cortisol	0.55	(0.43, 0.68)	

Table 7. Statistical results from ROC curves testing perceived stress and cortisol separately as predictors of ASD outcome. The model for perceived stress included the following covariates: maternal race/ethnicity, maternal age, and home ownership. The model for cortisol included: maternal race/ethnicity, maternal age, home ownership, creatinine, and number of thaws for the urine sample aliquot. P-value was calculated by fitting both stress measures in one model with all covariates for the individual models.

Table 8. Results of sensitivity analysis—trimester natural log transformation of cortisol

Results of trimester natural log transformation of cortisol						
	ASD (vs. TD)			**Non-TD (vs. TD)**		
Time Point (total n)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**
Trimester 1 (n=112)	27	0.58 (0.10, 3.38)	0.54	18	0.36 (0.04, 3.19)	0.36
Trimester 2 (n=99)	25	3.73 (0.63, 22.19)	0.15	14	0.49 (0.05, 4.56)	0.53
Trimester 3 (n=47)	11	0.25 (0.02, 2.59)	0.25	5		
Pregnancy Average (n=112)	27	0.71 (0.10, 5.34)	0.74	18	0.14 (0.01, 1.65)	0.12

Table 8. Results from multinomial logistic regressions of cortisol scores in each trimester, adjusted for covariates for the sensitivity analysis. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and non-TD groups, with TD as the reference group. The Pregnancy Average timepoint reflects across all three trimesters. Relative Risk Ratio for non-TD in the third trimester is not presented due to small sample size.

Table 9. Results of sensitivity analysis—sex-specific estimates of neurodevelopmental outcome for ln cortisol in ASD vs. TD

Female							
	ASD (vs. TD)			Non-TD (vs. TD)			
Trimester (n=total)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	Interaction p-value
Trimester 1 (n=62)	9	0.34 (0.05, 2.24)	0.26	10	0.37 (0.04, 3.35)	0.38	
Trimester 2 (n=52)	7	2.80 (0.42, 18.71)	0.29	7	0.46 (0.05, 4.51)	0.51	REF
Trimester 3 (n=23)	3			3			
Male							
	ASD (vs. TD)			Non-TD (vs. TD)			
Trimester (n=total)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value	Interaction p-value
Trimester 1 (n=84)	27	0.50 (0.08, 3.01)	0.45	11	0.38 (0.04, 3.33)	0.38	0.18
Trimester 2 (n=73)	24	4.16 (0.64, 27.24)	0.14	9	0.54 (0.06, 5.05)	0.59	0.19
Trimester 3 (n=26)	9			2			

Table 9. Results from adjusted multinomial logistic regressions of ln cortisol in each trimester, adjusted for covariates for the sensitivity analysis to compare ASD vs. TD with an interaction term for ln cortisol and child sex. Total n's, sex-specific relative risk ratios, 95% confidence intervals, and p-values are presented. Associations in trimester 3 were not examined due to small cell sizes.

Table 10. Results of sensitivity analysis—trimester natural log transformation of cortisol deviations

Results of trimester natural log transformation of cortisol deviations						
	ASD (vs. TD)			Non-TD (vs. TD)		
Time Point (total n)	n	Relative Risk Ratio (95% CI)	p-value	n	Relative Risk Ratio (95% CI)	p-value
Trimester 1 (n=112)	27	0.84 (0.44, 1.59)	0.58	18	1.14 (0.55, 2.38)	0.73
Trimester 2 (n=99)	25	0.71 (0.32, 1.57)	0.4	14	0.97 (0.33, 2.80)	0.95
Trimester 3 (n=47)	11	0.37 (0.06, 2.39)	0.29	5		
Pregnancy Average (n=112)	27	0.76 (0.39, 1.52)	0.44	18	0.96 (0.43, 2.14)	0.92

Table 10. Results from multinomial logistic regressions of cortisol in each trimester, adjusted for covariates in the sensitivity analysis. Total n's, relative risk ratios, 95% confidence intervals, and p-values are presented for ASD and non-TD groups, with TD as the reference group. The Pregnancy Average timepoint reflects across all three trimesters. Relative Risk Ratio for non-TD in the third trimester is not presented due to small sample size.

Table 11. Results of sensitivity analysis—race-specific estimates of neurodevelopmental outcome for ln cortisol

non-Hispanic white							
	ASD vs. TD			Non-TD vs. TD			interaction p-value
Trimester (n=total)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	
Trimester 1 (n=78)	21	0.21 (0.03, 1.65)	0.14	12	0.10 (0.008, 1.21)	0.07	
Trimester 2 (n=65)	19	1.75 (0.19, 16.23)	0.62	9	0.45 (0.03, 7.81)	0.58	REF
Trimester 3 (n=22)	7	0.08 (0.003, 1.82)	0.11	1			
Other							
	ASD vs. TD			Non-TD vs. TD			interaction p-value
Trimester (n=total)	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	**n**	**Relative Risk Ratio (95% CI)**	**p-value**	
Trimester 1 (n=68)	15	2.01 (0.21, 19.03)	0.54	9	1.44 (0.10, 19.99)	0.78	0.05
Trimester 2 (n=60)	12	7.70 (0.78, 71.53)	0.08	7	0.56 (0.04, 7.16)	0.66	0.57
Trimester 3 (n=27)	5	0.36 (0.03, 5.02)	0.45	4			0.12

Table 11. Results from adjusted multinomial logistic regressions of ln cortisol in each trimester, adjusted for covariates to compare ASD vs. TD and Non-TD vs. TD in the sensitivity analysis. Total n's, race-specific relative risk ratios, 95% confidence intervals, and p-values are presented. RRRs were calculated from one model, which included an interaction term for PSS score and maternal race. Associations for Non-TD vs. TD in trimester 3 were not examined due to small cells sizes.

Conclusion

The true etiologies of ASD and Non-typical development are still unknown, but many genetic and environmental studies have contributed to answering this question. This study had an overall objective of examining the association of prenatal maternal stress and child neurodevelopmental outcome. To date, it is the first study to investigate this association in a high familial risk cohort. Prenatal stress was measured in three different ways and presented in three separate chapters. In Chapter 1, stress was measured as mothers experiencing any of 17 stressful life events listed. In Chapter 2, stress was measured as perceived stress by examining scores from the Perceived Stress Scale. Finally, in Chapter 3, stress was measured by prenatal cortisol concentrations from 24-hr urine collections.

In Chapter 1, it was found that experiencing legal problems, including immigration problems, was associated with higher risk of Non-TD, compared to TD. All other SLEs in association with ASD and Non-TD outcome were null, which may be explained by the fact that this study population was a high familial risk cohort. With mothers already having at least one child diagnosed with ASD before this MARBLES pregnancy, they have already had experience navigating all that this entails, which includes (but is not limited to) locating services for a diagnosis for her child, receiving and understanding the diagnosis, finding services for her child, learning new ways to interact with her child, etc. These mothers could be more resilient at handling stressful life events since their baseline stress levels might be higher compared to mothers that do not already have a child diagnosed with autism. Alternatively, it may not necessarily be the stressful events experienced themselves, but how the event is perceived by the mother that is associated with risk, which is what associations show in Chapter 2.

In the second chapter, higher perceived stress scores were associated with higher relative risk for Non-TD in the first trimester and with higher relative risk for ASD in the second and third trimesters. This finding and the result that perceived stress had the biggest increased relative risk in the second and

third trimesters are similar to previous investigations' findings indicating that this time period of pregnancy is associated with the greatest risk.[22,23,90] Although the mechanisms behind any associations between MPS and neurodevelopment warrant future research, the timing for a critical period for stress in the second half of pregnancy is in line with a common theory of the way maternal stress affects the fetus through the HPA axis. While the fetal hypothalamus begins forming around 9-10 weeks gestation, it is not fully able to function until early in the second trimester of pregnancy.[44] It is theorized that during the second trimester, a mother's prenatal stress may begin to be experienced by the fetus, which could alter the fetus's HPA axis function to a higher set point or greater reactivity, which in turn suppresses the fetus's immune response, resulting in atypical development.[47] Once the HPA axis is developed, the fetus would experience the effects of the mother's stress in the second and third trimester.

The authors of this present study examined perceived stress, with the rationale being that it may not be the stressful life events experienced themselves that could be associated with an increased risk to the child, but instead how the mother perceives these events. Additionally, there are also everyday stressors that are not considered when solely focusing on stressful life events. For example, everyday work stressors or the holiday season are not something that would be considered a major stressful life event but undoubtedly have an effect on a person. Perceived stress is a way to measure these non-major life events that still result in stress on the mother. Thus, the perceived stress score was used, and these significant findings confirm the hypothesis that perception of stress is a risk factor, whereas previous work suggests stressful events themselves are not.

In Chapter 3, prenatal urinary cortisol was not found to be associated with neurodevelopmental outcome. The sample size for this study was relatively small, thus it is highly possible that smaller effects were unable to be detected, resulting in non-significant findings. In the second trimester, increased

cortisol was associated with increased risk of ASD, though not statistically significant. In first trimester analyses, increased cortisol was associated with decreased risk of ASD and Non-TD, but again, non-significant with low precision. However, the general direction of findings in this study agree with another prospective study conducted which found an association between lower maternal cortisol and increased ASD symptoms in the child.[33] Decreased cortisol during pregnancy and elevated risk of ASD could be explained by mothers having a decreased reaction to stressors due to chronic stress. This chronic stress would alter HPA-axis function[98] so her change in cortisol levels from additional stressors might be lower than expected. This explanation would be in agreement with existing studies that have found that mothers of children with ASD had decreased awakening cortisol levels compared to mothers of children without ASD.[99,100]

Overall, this study's findings suggest that it may not be the stressful life events themselves experienced during pregnancy or how the mother's body biologically responds to these stressors, but instead how they perceive these events to be stressful that may correlate with risk of ASD or Non-TD outcomes in the child. Stress reduction intervention could serve as preventative measures that help optimize the child's long-term neurodevelopmental health in high familial risk families.

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