

2015

Temporality of Risk Factors and the Gender Differential Related to Autism Spectrum Disorder Diagnosis

Donna L. Sullivan
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Walden University

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Donna Sullivan

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Walden University

2015

Abstract

Temporality of Risk Factors and the Gender Differential

Related to Autism Spectrum Disorder Diagnosis

by

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MBA, Saint Louis University, 1993

MS, University of Minnesota, 1990

BA, University of Minnesota, 1982

Dissertation Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

Public Health, Epidemiology

Walden University

February 2015

Abstract

Autism spectrum disorders (ASD) constitute life-long neurodevelopmental conditions. Globally, ASD risk for males remains 2 to 4 times greater than for females. Critical exposure mechanisms, their timing on ASD risk, and associations with the ASD gender differential remain elusive. The purpose of this study was to describe the relationship between preconception, pregnancy, recalled lactation practice, and infant traits, on ASD risk, and the gender differential of ASD. A recently published temporal framework was adapted to study effects of maternal smoking and vitamin use, and recalled lactation practice on offspring ASD diagnosis with adjustment for preconception health and infant breathing traits. A retrospective case-control analysis using 733 child data records from a U.S. autism registry that contained familial and nonfamilial controls characterized child gender-stratified relationships of 9 study variables. Logistic regression results showed prior maternal smoking, male gender, and maternal recollection of lactation practices were associated with significantly higher odds of offspring ASD diagnosis. Exposure factors associated with ASD did not differ significantly by child gender or maternal vitamin use. Infant respiratory distress at birth was a covariate and collinearly related to obstetric risks. Maternal smoking was antecedent to respiratory distress and lactation practice. Study limitations included incomplete responses without repeated measures for recalled lactation practice and maternal diet variables. Implications for positive social change include a better understanding of reproductive, preconception, and prenatal risk factors of ASD. The study results have implications for reproductive health, smoking cessation programs, family planning, and prenatal care for women of reproductive age.

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Acknowledgements

I would like to thank my son, Michael, and his father, Tom, for sacrificing family time and taking on additional responsibilities as I spent endless days, nights, and weekends studying and writing. I hope Michael has learned the level of commitment and sacrifice involved in higher education as he begins his own college journey.

I would like to express gratitude and thanks to my committee chair, Dr. Angela Prehn, for her positive and encouraging feedback and for high standards of academic rigor. Dr. Prehn encouraged critical thinking and writing skills and challenged me to improve the logic arguments and writing flow. Thank you to Dr. Amany Refaat who provided guidance and direction and cautioned me about the unique challenges of using archival data. And I'd like to thank Dr. Jim Goes for improving the readability and writing quality of my dissertation.

I gratefully acknowledge the resources provided by the Autism Genetic Resource Exchange (AGRE) Consortium and the participating AGRE families. The Autism Genetic Resource Exchange is a program of Autism Speaks and is supported, in part, by grant 1U24MH081810 from the National Institute of Mental Health to Clara M. Lajonchere (PI).

Many people who touched my life 5 years ago as I started this quest to better understand autism. I had unexpected opportunities to share the joys, wonderment, heartaches, and frustrations of working with children who were the catalysts for my professional curiosity into the biological aspects of autism. I would like to thank them.

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Chapter 1: Introduction to the Study

The medical and educational terminology and criteria used to describe essential or early onset autism has changed over the years but continues to reflect a common observable outcome based on social, communicative, and repetitive behavioral observations (American Psychological Association, 1994; Centers for Disease Control and Prevention [CDC], 2007; Daniels et al., 2011; United States Department of Health and Human Services [DHHS], 2008). The increased global prevalence and public health costs of autism affect health care, education, and community health economics. Annual health care utilization costs within a U.S. nationwide sample were estimated to be \$5,000 more per year for children with ASD than health care utilization costs for typically developing children (Liptak, Stuart, & Auinger, 2006). A more recent estimate suggested medical costs alone may be \$49 billion annually, with families spending \$67,000 per year in medical, educational, and psychological therapy to care for an autistic child; this accumulates to an estimated \$3.2 million over the lifetime of the person with autism (Saunders, 2010).

As early as 1968, epidemiologists began to suspect autism was associated with biological rather than psychological dysfunction (Rutter, 1968). Genomic research suggests that more than 80 to 85% cases cannot be directly attributed to particular genetic risks (Bukelis, Porter, Zimmerman, & Tierney, 2007; Hallmayer et al., 2011; Miles, McCathren, Stichter, & Shinawi, 2010). Despite the increased number of cases of ASD over time globally and within the United States, the gender differential has consistently been reported to be approximately 4 times higher for males (Elsabbagh et al., 2012) for

diagnostic criteria used by psychologists, physicians, and educational professionals (Daniels et al., 2011; DHHS, 2008).

ASD comparative pathobiology associated with gene expression in nonaffected siblings suggested genes and metabolism are involved in the nervous system development, inflammation, and cytoskeletal organization (Hu et al., 2009). Organ and metabolic dysfunction within six biochemical pathways has been associated with ASD in children, but it is unclear if these are secondary symptoms or primary causes of ASD (Abrahams & Geschwind, 2008; Gabory, Attig, & Junien, 2009). Metabolic biomarkers for cellular stress and dietary exposures have been studied with inconclusive results. The influence of restrictive and selective dietary preferences and pharmacotherapy use by children with ASD confounds study designs and data interpretation.

ASD studies generally employ case-control designs for hypothesis testing, but few studies have reported on gender differences among ASD cases. Study designs and research progress are limited by the rarity of the condition, especially among females, which complicates study of the ASD gender differential. There are also few theories about causative roots the of ASD gender differential.

Large population based studies containing over 500 cases and case control studies have shown gender-associated relationships between parental age, fetal distress, induced labor, and maternal health status (Burstyn, Wang, Yasui, Sithole, & Zwaigenbaum, 2011; Croen et al., 2007; Dodds et al., 2012; Gregory, Anthopolos, Osgood, Grotegut, & Miranda, 2013; Habek & Kovacevic, 2011; Krakowiak et al., 2012; Mann, McDermott, Bao, Hardin, & Gregg, 2010). The relative contribution of these prenatal factors is a core

focus of etiological research. However, even for large population based studies, a case-control design is ineffective for investigating temporality of risk factors (Colditz, 2010; McDonald & Paul, 2010). The critical molecular, epigenetic, or biological exposure mechanisms, gene-environment interactions, the impact of their timing on ASD onset, and the ASD gender differential remains largely a scientific mystery.

Therefore, it is of epidemiologic interest to understand if the ASD gender differential may inform the root causes of ASD. Research on gender-associated metabolic differences are lacking in ASD cohorts. In addition, there is need for a unifying, temporal framework of reproductive factors.

Observable, consistently detailed medical records and readily documented gender-based factors may provide robust analysis of the effect of reproductive and prenatal factors on the gender differential of autism. Retrospective, externally validated prenatal risk factors, including parental age, preconception health status, parental risk behaviors, pregnancy and delivery complications, and familial genetics are likely associated with ASD risk. Insights into shared and unique risk exposures and the timing of such exposures during fetal and child development may also inform ASD risk profiles for subsequent offspring. Such interrelated factors may also inform the gender-differential risk associated with autism onset. The gender differential in autism is generally presumed in the literature to be biologically and possibly hormonally based, but this area of etiologic research deserves more attention to better understand the role of preconception health, prenatal health, and epigenetics in the development of ASD risk.

In the remainder of this chapter, I present the exposure, genetic, and epigenetic literature background for the theoretical construct of a temporally-constructed framework to study ASD risk factors, and the ASD gender differential. The concepts of biological susceptibility and deterministic fetal programming, further articulation of the etiological problem, the purpose of my dissertation, and a hierarchical framework to study essential research questions will be described in this chapter. The framework is an adapted temporal model from previous population research on ASD etiology but includes hypotheses about possible epigenetic theories of ASD.

Background

Preconception, prenatal and perinatal gene-environment mechanisms, and subsequent changes in metabolic processes may be integral to ASD etiology. Suggestive, replicated evidence of altered metabolic processes that reflect a comprehensive profile of metabolic dysfunction has been characterized for autism (Abrahams & Geschwind, 2008; Gabory et al., 2009). However, these theories have not integrated the temporality of fetal risk factors. Furthermore, reproducible biomarkers for autism have not been identified. Moreover, generalized causative mechanisms have not addressed the risk factors associated with infant gender or identified biomarkers of exposure. The proposed pathways have not theoretically accounted for the gender differential that is consistently reported across race, ethnicity, culture, genome, and parental health status. Additionally, temporality, uncertain etiology, and gender-stratified risk profiles are challenges to the characterization of autism onset and diagnosis. Hence, exposure analysis research for ASD risk is problematic. It is unclear if proposed biomarkers are symptoms or biological

indicators of ASD. It is also unclear whether the ASD gender differential is a genetic, hormonal, gene-environment, or social risk factor. Thus, the study of plausible epigenetic mechanisms may inform fetal exposure risk profiles. A more comprehensive, theoretical framework that reflects ASD etiology is needed.

Exposure Factors

Exposure-induced altered genetic and cellular metabolic processes may be measureable if proxy variables for these changes can be identified and quantified. Intermediate biologic markers for critical pathways, as proxy indicators of body burden, are typically modeled to estimate dose or outcome effect (Checkoway, Pearce, & Kriebel, 2004; Moeller, 2005). Biologic markers may be proxies for exposure, for metabolic uptake or “body burden,” or they may represent symptomatic or downstream metabolic effect indicators (Checkoway et al., 2004; Katzung, 2001). Biomonitoring indicators may not reflect cumulative dose or may be highly variable over time or between subjects. Individual genetic traits, past exposures, lifestyle, and health status of study participants may confound the relationship through individual susceptibility (Belsky & Pluess, 2009; Miles et al., 2010; Taylor & Rogers, 2005; Teschke et al., 2002).

Various blood biomarkers have been extensively studied in children diagnosed with ASD, but most studies were complicated by the lack of comprehensive characterization of prenatal health factors, pregnancy history, and unique social and biological developmental trajectories of affected children and families (Lauritsen, Pedersen, & Mortensen, 2005; McDonald & Paul, 2010). Studies reporting the use of metabolite biomarkers were complicated by current dietary practices and pharmaceutical

therapy for affected children who were predominantly male (Adams et al., 2011; Evans et al., 2008; Nikolov et al., 2008; Rosenberg, Landa, Law, Stuart, & Law, 2010; Taurines et al., 2010). There has been very little clarification in biomarker research regarding the discernment of timing, causes, and symptoms of autism or the gender differential of ASD (Kumbier, Dudley, & Thome, 2010; Ratajczak, 2011).

Due to temporal factors, there are concerns that biomarker candidates for autism may be clinical manifestations or symptoms of the condition, rather than critical proxy variables of causation. Autism diagnosis is often validated after a child's social and language skills have matured and stabilized, typically at age 8, in order to minimize diagnostic variability and bias (CDC, 2012; Daniels et al., 2011; Posserud, Lundervold, & Gillberg, 2009). Therefore, prenatal and early life exposures may not be well characterized or documented in anticipation of potential subsequent diagnosis at age 8 calendar years. For ethical reasons, prenatal or genetic screenings for autism and for gender-based diagnostic risk are ill-advised due to concerns over test sensitivity and specificity (Miles et al., 2010; National Human Genome Research Institute [NHGRI], 2011; Wilfond & Ross, 2009).

Vaccinations containing thimerosal are often reported in the popular press to be a key ASD risk factor. However, the reported associations of ASD and childhood vaccine or thimerosal exposure are not adequately supported by current scientific evidence (DeStefano & Thompson, 2004; Makela, Nuorti, & Peltola, 2002; Poland, 2011). In addition, there does not appear to be a direct relationship between autism and inflammation-induced cytokines or between ASD and reactive immunoglobulin

expression in infants or children (Goines et al., 2011; Onore, 2009; Rosen, Yoshida, & Croen, 2007). Despite observable tendency to encephalitic outcomes in ASD toddlers (Schumann, Barnes, Lord, & Courchesne, 2009) and documented sulfation pathway anomalies in ASD children (Campbell et al., 2009; Erickson et al., 2005) there are few stable cytokine, dietary, or urinary biomarkers that can be studied in association with ASD (Kidd, 2010; Ratajczak, 2011; Wilson, 2014).

There are also few, if any, identified unifying, validated, or conclusive childhood predictive ASD biomarkers of shared or unique environmental exposures (Zerbo, Iosif, Walker, Ozonoff, Hansen, & Hertz-Picciotto, 2013). Comorbidities and confounding interactions related to childhood food allergenicity and selective and repetitive food selection among autistic children are common (Genuis & Bouchard, 2009; Johnson, Handen, Mayer-Costa, & Sacco, 2008). Furthermore, many commonly administered pharmacotherapies for children with autism affect developing digestive processes, metabolism, hormones, nutrient uptake, and biomarker composition (Palmieri, Papeleo, Porcelli, Scarcia, & Gaita, 2010; Schultz et al., 2008). However, the inability to identify reproducible ASD biomarkers may also be due to possible confounding effects of genetic expression or allele profiles on metabolism (Cheslack-Pestova et al., 2007; Nijmeijer et al., 2010; Schmidt et al., 2011; Wilhelm-Benartzi et al., 2012).

Only a few research papers were identified that addressed hypothesized mechanisms, exposures, or biomarkers that may be related to gender-differentials of ASD (Adams et al., 2011; Ashwood et al., 2008; Default et al., 2009, 2012; Evans et al., 2008; Hu et al., 2009; Lauritsen et al., 2005; Nikkila et al., 2008; Pan, Ober, & Abney, 2007;

Pastural et al., 2009; Wiest, German, Harvey, Watkins, & Hertz-Picciotto, 2009). The gender differential of ASD, however, is a well known fact. Few papers proposed mechanisms and simultaneously quantified related biomarker associations for the gender-differential in children with ASD. Therefore, gender-specific metabolic biomarker research among healthy males and female infants and children has been identified as an emerging area of autism study. Significant gender differences in serum biomarkers were identified in healthy infants at age 1 (Nikkila et al., 2008), and earlier research suggested X-linked chromosome or epigenetic effects may influence biomarkers such as lipoprotein and tryglyceride levels (Pan et al., 2007).

Because of uncertain etiology, indeterminate biomarker monitoring, and disparate timing of onset and diagnosis, autism research designs tend to be ecological and retrospective in nature. Most are observational studies based on an environmental epidemiological framework, with presumed exposure profiling designed to test the degree of association, magnitude, and direction between exposure and outcomes. Challenges arise in assessing the current autism etiology research literature due to the uncertain timing of exposures that may include secular change and multiple, diverse, confounding, or mediating environmental, chemical, or biological agents.

Genetic Factors

Historically, the persistent gender prevalence ratio risk for other developmental disabilities was attributed to genetic variability (perhaps X-chromosome or gene related) expressed in females only when pathological (i.e., both X-genes affected). Random genetic mosaicism or genetic diversity of human females is reportedly higher than males

(Gabory et al., 2009, Pan et al., 2007; Ober, Loisel, & Gilad, 2008). However, offspring statistics on ASD-positive mothers have not shown an increased reproductive risk of subsequent onset autism diagnosis (Goin-Kochel, Abbacchi, & Constantino, 2007; Pickles et al., 2000). Thus, ASD inheritance mechanisms attributed to dominant X chromosome (i.e., X-X genotype traits) are not directly associated with autism diagnosis among offspring. However, it is plausible that other genetic factors, such as random, Mendelian gene-gene interactions may also contribute to the risk of autism onset. Genome-wide analysis techniques have been used to study the relationship among gene transcription patterns, familial genetic profile characterizations, and subsequent autism diagnosis in offspring.

More than 40 published genome-wide association studies for autism have been reviewed with suggestive but inconclusive results (Miles et al., 2010). Even among monozygotic and dizygotic twins studies, concordance rates for autism diagnosis have been reported to be in the range of only 60 to 82% (Hallmayer et al., 2011; Hu, Frank, Heine, Lee, & Quackenbush, 2006; Lauritsen & Ewald, 2001; Rai, 2010; Sharp et al., 2011). This low concordance suggests genetic risk is not equally shared, even among twins. Hu et al. (2009) used a case-control like design for gene expression profiling among twins and nonaffected sibling pairs and speculated cholesterol/steroid metabolism and androgenic hormone levels may affect the gender differential pathobiology of autism. Low concordance in twin studies provided evidence for the hypothesis that chromatin reversion and/or epigenetic mechanisms may be at least partially responsible for subsequent onset autism (Chao et al., 2010; Gibson et al., 2010). Genetic studies

designed to investigate sex-specific autosomal or X-gene-linked effects have not improved statistical power for the replication of high risk group profiles (Carayol et al., 2011), even when using innovative techniques such as sequential oligogenetic linkage analysis routines- SOLAR (Kent, Dyer, & Blangero, 2005; Kent et al., 2005). Therefore, gene transcription profiling has not been implicated as a fundamental mechanism related to autism onset.

The consequences of assisted reproductive technology with regard to alteration in genetic material, chromatin reversion, cytosolic environment, and genetic function and expression continue to be better understood, but they likely also affect genetic susceptibility and fetal programming. Furthermore, the prevalence of assisted reproductive technology is not consistently measured in the United States due different reporting systems used in vital records, clinical records, and federally supported surveillance programs (Barradas et al., 2012).

Increased parental age may be associated with chromosomal damage or assisted reproductive technology (Jenkins, 2013; Schieve et al., 2011). Genetic susceptibility due to chromosomal damage or preexisting maternal conditions may have different relative risk contributions to ASD than do epigenetic mechanisms associated with placental transfer mechanisms. However, these factors and epigenetic theories have not been adequately studied to provide insight into ASD risk and the gender differential of ASD.

Gene function and expression may be dynamically affected through gene-environment interactions, which may affect ASD onset. The relationship of gene-environment interactions and autism is an emerging field of etiological study. Several

plausible concepts have been proposed to describe the interaction of genetic and environmental exposure factors, also referred to as epigenetic interaction factors. A graphic summary of noteworthy genetic, parental, and child risk factors associated with ASD is shown in Figure 1. The temporality, interaction, and directional association of these factors is not well-understood. Hence, there is need to further study the temporal sequence of ASD mechanisms.

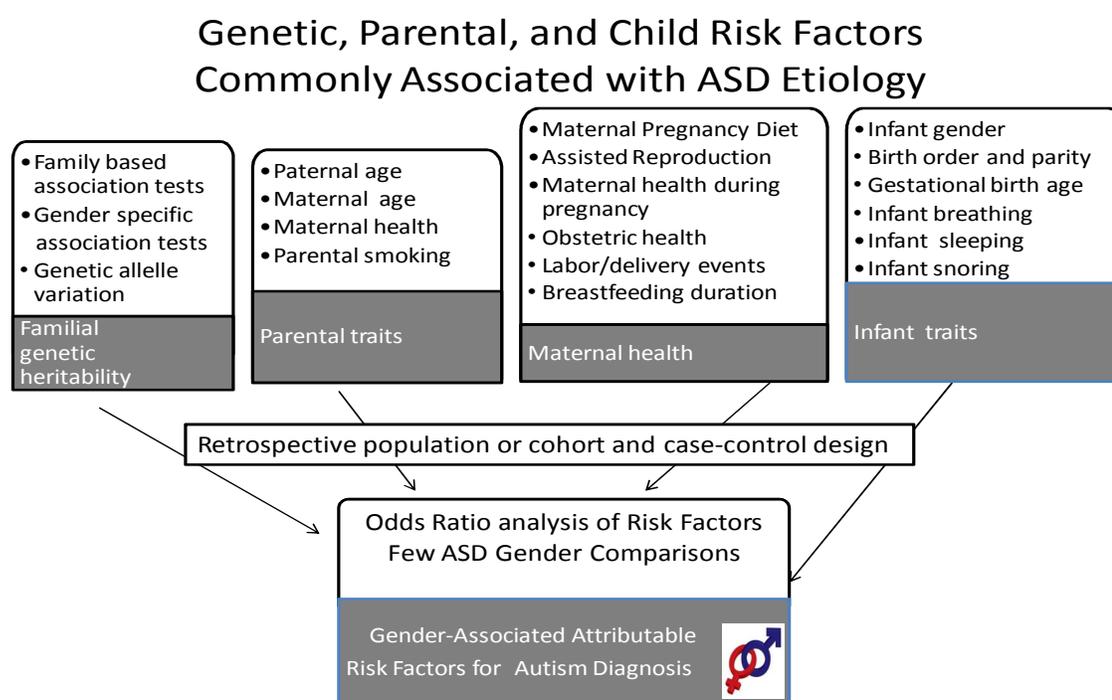


Figure 1. Genetic, parental, and child exposure factors associated with ASD.

Epigenetic Theories

There are two unifying concepts that are generally proposed to rationalize the sequence of events that may result in adverse offspring consequences, including childhood disorders and autism risk. These concepts have been expanded and are described below.

Biological susceptibility was first described by Wade Hampton Frost in 1937 with regard to chronic disease conditions (Frost, 1977). Subsequent refinement of the concept was offered by the concept of *multifactor liability threshold* (Tsia et al., 1981), which describes the impact of multiple, weak associations of risk factors associated with autism onset and diagnosis. The ASD liability was presumed to be normally distributed, but individuals would not be affected unless environmental factors exceeded a certain threshold level. While Tsia et al. (1981) described the theorized mechanism as more complex than the classic environmental exposure-dose-disease model, the researchers did not address genetic variability, temporal factors of exposures, or childhood developmental risk profiles that may be associated with autism.

Barker and Connor (1986) first described the concept of *fetal programming* with regard to chronic disease outcomes and suggested that trigger mechanisms for later life conditions such as cardiovascular disease were strongly influenced by the fetal environment. The concept of *fetal programming* has been expanded to consider a dynamic interaction between fetal development and growth and unique and fluctuating maternal health status (Finney-Brown, 2011; Lillycrop, 2011). Recent research in placental transport and physiology has provided evidence that fetal programming may provide an explanatory mechanistic framework for the trigger events and consequences that affect fetal development (Salafia, 2011; Sibley, 2009). However, the impact of genetic variability on the concept of *fetal programming* is not well known.

There is no consensus on the definition of *genetic susceptibility* to ASD, and genomic studies have been inconclusive. Dodds et al. (2011) used the term to represent an ASD

case subject having an affected sibling or mother with a history of psychiatric or neurologic condition.

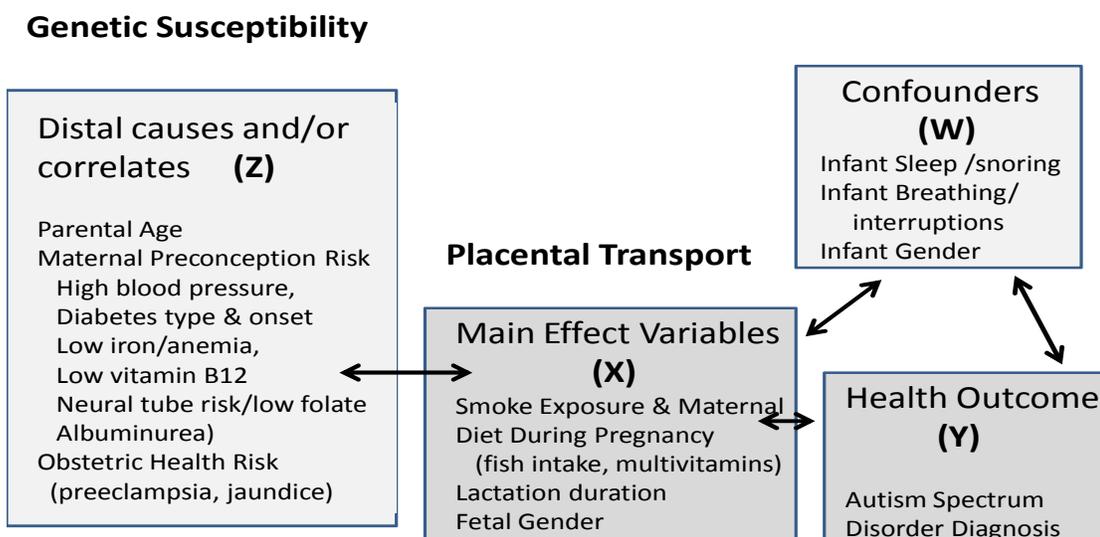
Therefore, among a well characterized genomic cohort, a focus on preconception health, well-documented prenatal factors and early life exposures may provide more study control over pivotal risk factors and exposure profiles associated with initial autism diagnosis at age 8. Expansive, prospective cohort studies, such as the *Earlistudy* funded by the National Institute of Health and the advocacy group Autism Speaks, have recently been initiated to clarify the effects of certain prenatal and perinatal risk factors implicated in autism (as cited in Newschaffer et al., 2012).

Critical windows of prenatal exposure to chemical agents such as tobacco smoke have been documented (Gardener, Spiegelman, & Buke, 2011; Gray, Eiden, Leonard, Shisler, & Huestis, 2010) and provide suggestive evidence to support the *biological susceptibility* of multiple weak environmental assaults and/or the more deterministic *fetal programming* concepts that may apply to autism onset and the ASD gender differential (McDonald et al., 2006). Common gender-associated infant trait risk profiles, respiratory dysfunction such as fetal hypoxia and distress, induced labor, and overlapping exposures factors associated with sudden infant death and nonregressive autism have been reported in the literature with little etiologic explanation (Dodds et al., 2011; Elsabbagh et al., 2012; Gregory et al., 2013; Kinney & Thatch, 2009; Kolvezon, Gross, & Reichenberg, 2007).

The purpose of this study was to investigate the relationship between preconception health, obstetric health, prenatal health, maternal smoking and nutrition,

lactation duration, and infant breathing and sleeping patterns and ASD in a U.S. genomic cohort. Using a narrowed focus on early life, prenatal exposures, and closed-system or captively controlled dietary intake (prior to the use of solid foods) within a genomic cohort, I aimed to study the relationship of parental traits, pregnancy onset factors, and neonatal behavior to autism diagnosis to explore the gender risk differential in autism. Using case-control methodology, the association of pregnancy factors (maternal smoke exposure, multivitamin use, and lactation) to ASD outcome was explored, with consideration of the effect of preconception parental age, preconception maternal health status, obstetric complications, and neonatal traits. These exposure-timing relationships were studied with stratification of infant gender and compared between cases and a diverse control group within an autism family genomic registry cohort.

Proxy exposure variables associated with direct placental exchange theory were proposed to relate to higher odds ratio of autism diagnosis. Thus, fetal exposure risks during pregnancy and lactation may suggest significant main effects in the overall exposure risk relationship and ASD. It was of interest whether direct placental exchange mechanisms may inform the gender differential of ASD between boys and girls. A proposed framework for the study is illustrated in Figure 2. The biological rationale for the ASD gender differential has not been studied extensively, and the pathobiology deserves additional research attention. Gender-stratified observational study results may improve understanding of autism etiology, inform future prospective cohort study designs, and validate reproductive and maternal health education practices, potential preconception, prenatal behavioral, and dietary interventions.



The hypothesized hierarchical relationship between distal preconception risk factors (Z), direct placental fetal exposures (X), and neonatal infant sleep and breathing patterns (W) and autism diagnosis (Y). Adapted from Figure 1. and Burstyn, Wang, Yasui, Sithole, & Zwaigenbaum 2011

Figure 2. Proposed temporal and epigenetic theory of exposure factors and ASD.

Problem Statement

Uncertain timing and impact of exposures and gene-environment interactions complicate ASD etiological research (McDonald & Paul, 2010). For example, reproducible suggestive evidence exists for physiologic differences in nervous system development, inflammation, and cytoskeletal function among ASD and typical developing children (Abrahams & Geschwind, 2008; Gabory et al., 2009; Hu et al., 2009). Additionally, three epigenetic and six altered metabolic processes have been characterized for ASD (Abrahams & Geschwind, 2008; Gabory et al., 2009). However, etiologic mechanisms have not addressed the exposure-timing aspects of genetic

susceptibility versus environmental exposure risks associated with ASD, the relative contribution of epigenetics versus exposure-dose on the gender differential of autism.

There is a persistent gender risk differential in ASD onset between boys and girls across race, ethnicity, culture, and genome (Elsabbagh et al., 2012). The medical, biological, or biochemical rationale for the 4-fold predominance of male autistic children compared to females has not been deeply explored but has been accepted as a social or biological norm. There is also a significant gap in the medical and epidemiological literature regarding plausible hypotheses that may help elucidate the rationale for trebled or quadrupled clinical autism prevalence ratio of males to females.

In addition, there remains a lack of unifying or hierarchical framework for the study of ASD risk factors. The plausibility of direct placental exchange or *fetal programming* mechanisms applied to ASD or the gender differential deserves more attention (Lillycrop, 2011; Myatt, 2010; Wilhelm-Benartzi et al., 2012). Moreover, the discovery of plausible genetic or cellular mechanisms that may impact ASD risk and the ASD gender differential is not well studied. There are substantial uncertainties about genetic and epigenetic risk mechanisms of ASD onset. Research regarding biomarkers and metabolic profiles for healthy persons stratified by gender is just emerging as a focus of metabolomic study (Mittelstrass et al., 2011; Nikkila et al., 2008; Weiss, Pan, Abney, & Ober, 2006). Metabolomics as a function of exposure-timing may inform the gender differential consistently reported for subsequent autism diagnosis.

A study design of plausible, prioritized, temporally clustered prenatal, early life exposures and parental behavioral factors associated with the gender differential in

autism prevalence was constructed. The framework presumed genetic susceptibility reflected preconception gene function and expression, which may be influenced by parental use of assisted reproductive technology. However genetic factors were a secondary consideration in this study. Gene expression and epigenetic mechanisms, presumed to be associated with maternal exposures and direct placental transfer and *fetal programming* mechanisms, were hypothesized to be associated with temporality of ASD risk factors. Figure 2 reflects hypothesized relationships of epigenetic and temporal theories for ASD.

Purpose and Nature of the Study

The purpose of the study was to describe relationships between three pregnancy-related exposures and ASD outcome, as mediated by preconception health, prenatal health, obstetric complications, and neonatal traits, stratified by infant gender. Using a well-characterized genomic cohort, the study contributed to the limited body of research on the hierarchical relationship and temporality of factors, shared familial environmental risks, unique pregnancy related exposures, and possible confounding risk factors, such as infant gender, with ASD risk.

A retrospective, observational, case-control design was designed using a well-characterized population and diverse controls based on several previous Autism Genetic Resource Exchange (AGRE) cohort studies (Anello et al., 2009; Cantor, Yoon, Furr, & Lajonchere, 2007; Carayol et al., 2011; Cheslack-Postova et al., 2007; Hallmayer et al., 2011; Hu et al., 2009; Lu & Cantor, 2012; Martin & Horriat, 2012; Stone, Merriman, Cantor, Geschwind, & Nelson, 2007; Strom, 2010; Strom et al., 2010; Wallace,

Anderson, & Dubrow, 2008). The categorical or binary dependent variable was clinical autism diagnosis based on *DSM-IV* or ICD-9 (ADIR) criteria documented in AGRE case registry records. The categorical independent variables included preconception parental age, preconception maternal health (assessed by high blood pressure, diabetes, anemia/maternal iron deficiency, vitamin B12 deficiency, neural tube defect [low folate] risk, albuminuria status), obstetric complications (assessed by preeclampsia, jaundice delivery), maternal smoke exposure and diet (fish intake, maternal multivitamin use) during pregnancy trimesters and lactation, and neonatal infant breathing and sleeping patterns per medical record history data bases for families enrolled in the AGRE registry.

The AGRE registry contained family-based medical records for ASD-affected children and unaffected siblings (Lajonchere, 2012). The focus of the AGRE registry research has been on genetic blood sampling and analysis; however, additional attention is focused on psychometric analysis of clinical phenotype records (R. Butler, personal communication, August 7, 2013). Phenotypic medical records were archived by AGRE and included documentation of case definitions for complimentary and partially redundant parental self-report and physician-recorded survey items, ASD onset date and age, well defined exclusion criteria related to single gene disorders, assisted reproductive technology (ART) use, and regressive forms of autism. The use of coded data helped ensure blinded use of the AGRE open access registry population datasets of clinical data for characterized familial genetic profiles and familial history records.

Research Questions and Hypotheses

In this dissertation, I proposed a framework for prioritizing plausible early life exposures that may be associated with ASD, as affected by shared environment, gender, and genomics. This research studied the relationship of proxy, preconception, pregnancy, and early life exposure variables on ASD diagnosis and the ASD gender differential. The research questions addressed exposure-timing relationships of pregnancy factors to ASD and the gender differential of ASD. The relationship between pregnancy factors and ASD was adjusted for hypothesized and posthoc identified confounding neonatal factors and theorized covariates of preconception risk factors. Based on the independent variables of maternal smoke exposure and diet during pregnancy, lactation, and gender and their combination, subhypotheses were proposed. The specific study question and hypotheses are stated below for ASD outcome, with theorized confounders and covariates described in detail. Presumed covariate preconception factors and confounding infant traits were analyzed independently and in combination to inform the collinearity of relationships to ASD and the ASD gender differential risk.

The initial research questions addressed the exposure-timing relationship between pregnancy factors, individually or in combination and ASD outcome within the AGRE sample cohort. Secondly, it was of interest whether the relationship of pregnancy factors to ASD outcome was confounded by neonatal traits. The third series of questions tested whether preconception factors were effect modifiers of the relationship of pregnancy factors and ASD.

The initial three research questions addressed whether there was a statistically significant association between pregnancy factors such as maternal multivitamin use and direct maternal smoke exposure during pregnancy and exclusive lactation practice and the outcome variable, ASD diagnosis by ADIR criteria. These initial hypotheses presumed a primary, main effect relationship of pregnancy factors to ASD via plausible placental transfer mechanisms.

Research Question 1: What is the relationship between maternal smoke exposure before or during pregnancy and ASD risk in offspring within the AGRE cohort?

H_01 : There is no association between maternal smoke exposure and ASD outcome within the AGRE cohort.

H_a1 : There is a positive association between maternal smoke exposure and ASD outcome within the AGRE cohort.

Research Question 2: What is the relationship between maternal diet (fish and multivitamin intake) during pregnancy trimesters as ASD risk in AGRE offspring?

H_02 : There is no association between maternal fish and multivitamin intake during pregnancy and ASD outcome within the AGRE cohort.

H_a2 : There is an inverse association between fish and multivitamin intake during pregnancy and ASD outcome within the AGRE cohort.

Research Question 3: What is the relationship between lactation and ASD risk?

H_03 : There is no association between lactation and ASD risk in cohort offspring.

H_a3 : There is an inverse association between lactation and ASD risk in cohort offspring within the AGRE cohort.

After exploring the hypothesized primary relationship of pregnancy related variables to ASD outcome, the most robust and significant relationships were carried forward for further study. The potential confounding of neonatal traits and the effect modification of preconception risk factors was tested in subsequent analysis for ASD as defined by ADIR outcome criteria.

The fourth research question addressed whether neonatal sleeping or breathing traits confound the relationship of pregnancy factors to ASD outcome within the AGRE cohort.

Research Question 4: How is the exposure-timing relationship of pregnancy variables (maternal smoke exposure, diet, and lactation) to ASD confounded by neonatal infant sleeping or breathing traits when analyzed separately or in combination in the AGRE sample?

H₀4: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) and ASD outcome is not confounded by infant sleeping or breathing.

H_a4: The relationship between pregnancy exposures (maternal smoke exposure diet, and lactation) and ASD outcome is confounded by infant sleeping or breathing.

The fifth research question addressed whether infant gender mediated the effect of pregnancy related variables and ASD diagnosis among cases and controls in the cohort.

Research Question 5: How does the exposure-timing relationship of pregnancy variables (maternal smoke exposure, diet, and lactation) to ASD outcome differ by infant gender?

H₀5: The relationship of maternal diet, smoke exposure during pregnancy, and lactation to ASD outcome does not vary by infant gender.

H_a5: The relationship of maternal diet, smoke exposure during pregnancy, and lactation to ASD outcome does vary by infant gender.

The last three research questions addressed whether the relationship of pregnancy exposures (maternal smoke exposure and diet during pregnancy and lactation) to ASD varies by preconception parental age, preexisting maternal health, or obstetric risks.

Research Questions 6 through 8: How does the exposure-timing relationships between pregnancy exposure-timing variables (maternal smoke exposure, diet, and lactation) and ASD vary by preconception parental age, preexisting maternal health conditions, and obstetric risks?

H₀6: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome does not vary by preconception parental age.

H_a6: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome varies inversely by preconception parental age.

H₀7: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome does not vary by preconception maternal health.

H_a7: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome varies positively with preconception maternal health.

H₀8: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome does not vary by obstetric risks within the cohort.

H_{a8}: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome varies positively by obstetric risks within the cohort.

The purpose of this quantitative study was to describe the hierarchical relationship between pregnancy related risk factors to ASD outcome, the effect modification of preconception and confounding neonatal traits that may be associated with ASD and the gender-based diagnosis of autism. Categorical temporal clusters of archival data for variables representing preconception health, fetal exposures during pregnancy and lactation, and early life infant traits were compared among cases and controls and stratified by gender for a cohort of well-characterized familial genomes. Group comparisons between cases and controls and intraclass odds ratio analysis between genders was conducted while testing for main effects, covariates, and the interaction of study variables. Tests of association, tests of trends, gender-stratified odds ratios, covariate collinearity, and logit regression analysis were conducted.

Conceptual Framework

A hierarchical framework of ASD causal mechanisms may help to explain potential relationships among exposures and affected genetic, cellular, and metabolic processes in a fetus or infant. An exposure modeling framework seeks to address temporal factors, the specificity of key or critical assaults and coherent, plausible routes of disease progression, and clinical manifestation of the condition (Checkoway, Pearce, & Kriebel 2004; Seixas, Robins, & Becker 1993). Generalized pathways of metabolic dysfunction associated with autism diagnosis have been validated by many independent researchers, but uncertain etiology, indefinite critical windows of exposure, and necessity

of case-control study designs constraint the ability to identify biomarkers of ASD and characterize gender-differentiation of genetic, hormonal, and environmental factors affecting risk of ASD. These risk factor categories are further described in Chapter 2. Evidence of a coherent or comprehensive exposure modeling framework for the onset of autism is lacking. However, a study design framework that included distal, ecological, and neonatal factors hypothesized to be associated with autism was recently published (Burstyn, Wang, Yasui, Sithole, & Swaigenbaum, 2011) using birth delivery records and adjusting for maternal age within a cross-sectional study of Canadian populations.

The methodology of Burstyn et al. (2011) accounted for distal factors as well as a primary causal relationship and adjusted for confounding variables. The researchers developed an exposure algorithm that addressed the binary outcome of autism onset as a relationship among prenatal factors including fetal hypoxia and adjusted for socioeconomic status, birth year, and fetal gender. The research design was an example of a well-articulated conceptual framework of distal and critical or main effect exposure factors. Burstyn et al. (2011) also used an efficient logistic modeling to account for the multiple risk factors, and defined autism diagnosis as a binary outcome variable.

Distal Factors: Preconception Health and Obstetric Complications

An etiologic framework such as that used by Burstyn et al. (2011) may describe a critical path or the logical flow of effect among exposures and genetic, cellular, and metabolic processes (Creswell 2009; Trochim & Donnelly 2007). Relationships among these variables may be hypothesized to be coincidental or associated with identifiable, plausible mechanisms (Checkoway et al., 2004; Seixas et al., 1993). Dodds et al. (2011)

reported the role of obstetric and neonatal factors associated with ASD as defined by ICD-9 code 299 criteria differed by preexisting maternal disease state within a Canadian cohort. Gregory et al. (2013) reported similar results using vital statistics for a North Carolina population cohort. However, adjustment for the confounding effect of assisted reproductive technology was not discussed by Dodds et al. or Gregory et al.. In addition, infant congenital heart defects are often associated with chromosome X-linked disorders and particular genes such as ZIC3, HTX1, and HTX, which may be coincidentally associated with hypoxia and SIDS (Bailliard & Anderson 2009; Gioli-Perira et al., 2008). However, pediatric heart defects have also been associated with epigenetic mechanisms affecting delivery outcomes (American Academy of Pediatrics 2011; Zhu et al., 2007). Causative evidence of an association with SIDS and ASD is also lacking. Associations of SIDS and ASD may be mediated by distal risk factor effects (Dodds et al., 2011).

In this study, I proposed a framework for prioritizing plausible early life exposures that may be associated with ASD, affected by a shared environment, gender, and genomics (Figure 2). A multifactorial liability model was proposed to investigate multiple fetal assault risk factors via proxy variables for placental transport and fetal programming during pregnancy (smoke exposure and diet during pregnancy and lactation) as mediated genetic susceptibility (preconception parental age, preexisting maternal health, obstetric complications) and confounded by neonatal gender and sleeping and breathing traits within an AGRE cohort. The model was based on the previous temporal and statistical framework proposed by Burstyn et al. (2011) as described above. An adaptation of the framework is illustrated in Figure 2, as applied to

the proposed variables of this study. Obstetric complications were presumed to be genetically predetermined.

Main Effect Factors: Maternal Diet and Smoke Exposure, Gender, and Lactation

Proxy variables that reflect direct exchange of cells, gas molecules, or fluids through placental transfer or breast milk from mother to fetus or infant are hypothesized to have a direct or main effect on ASD risk. Critical human pregnancy exposures have been well documented (Dietert, Dietert, & DeWitt, 2011; Wilhelm-Benartzi et al., 2012). In this study, presumed *placental transport* mechanisms were represented by pregnancy onset variables of maternal smoke exposure, fish intake, and vitamin use during pregnancy and lactation. Preconception health factors such as maternal diabetes, high blood pressure, low iron/diagnosed anemia, Vitamin B12 deficiency, low folate intake, or albuminuria were hypothesized to be distally correlated with ASD and the gender differential of ASD occurrence. Obstetric complications assessed by diagnosed preeclampsia or jaundice delivery were proposed to mediate the relationship of pregnancy factors to ASD diagnosis. Assisted reproductive technology (ART) was associated with older parents, complicated pregnancies, obstetrics, and ASD risk (Schieve et al., 2011). ART use among parents with ASD offspring has not been studied extensively; however, Zachor and Itzchak (2011) reported ART use among families with ASD offspring tended to be higher (10.7%) than population prevalence (3.06%) in an Israeli sample. Parental age, low birth weight, and gestational age distributions did not differ by case-control status (Zachor & Itzchak, 2011).

Confounding Factors: Infant Sleeping and Breathing, Infant Gender

Infant breathing and sleeping patterns were presumed to be confounders, as they may represent underlying congenital heart defects, asthma, allergies, symptoms of psychotherapy medication, comorbid mental health conditions, or ear infections (Hartshorne et al., 2009). Furthermore, methodology and survey instruments for infant sleeping and sleep apnea traits are not standardized (Mahoney & Caterino 2011; Stewart & Amar 2013; Young, Dempsey, Peppard, Neto, & Hia 2009). These unrelated health conditions may not be directly impacted by placental transport pregnancy factors, fetal distress, hypoxia, or ASD associated risks. Sleeping position, asthma, infection, or congenital heart defects were presumed to be extraneous factors in the explanatory main effect factors of ASD. Infant gender was also defined as a confounder in this study in order to study statistical comparisons *ceteris paribus*.

Definition of Terms

In this study, the broad case definition of ASD was predefined by the AGRE registry inclusion criteria based on medical assessment of autism by the interactive interview (ADIR) described in Appendix A. The dependent variable criteria, ADIR score of 1, has been standardized and includes ASD and pervasive developmental disorders not otherwise specified (PPD-NOS) as the outcome variable. ADIR results for age-appropriate assessment were used.

Proposed main effect independent variables included archival categorical values of maternal smoke exposure during pregnancy, maternal diet during pregnancy, and lactation as measured by fish and multivitamin intake, lactation duration and dedication,

and fetal gender as described by parental recall and medical records collected by AGRE researchers (Cantor et al., 2007; Geschwind et al., 2001). Independent variables for the components of preconception health risk indices (parental age, maternal high blood pressure, diabetes, low blood iron/diagnosed anemia, vitamin B12 deficiency, low folate/neural tube defect risk, and albuminuria) and obstetric health risk (preeclampsia and/or jaundice delivery) have been defined and coded using a standardized questionnaires implemented by AGRE researchers (Cantor et al., 2007; Geschwind et al., 2001; Stone et al., 2004). Independent, presumed confounding variables of infant traits of sleep pattern and infant breathing interruptions have been defined by AGRE medical history questionnaires. Coding and possible categorical definition of variables and risk indices are described in Chapter 3, Table 4.

Biological susceptibility was first described by Wade Hampton Frost in 1937 to reflect a propensity for particular individuals to be more prone to risk of chronic disease conditions. The concept did not ascertain whether the individual risk was attributed to genes or random chance.

The *multifactor liability threshold* (Tsia et al., 1981) theory described the impact of multiple, weak associations of risk factors associated with autism onset and diagnosis. ASD liability was presumed to be normally distributed, but individuals would not be affected unless environmental factors exceeded a certain threshold level of overall risk contributed by complex mechanisms.

Fetal programming was defined by Barker and Connor (1986) to explain the relationship of chronic disease outcomes as being associated with exposures within the

fetal environment. The concept of fetal programming has been expanded to consider a dynamic interaction between fetal development and growth, and unique and fluctuating maternal health status (Finney-Brown 2011; Habek & Kovacevic 2011; Lillycrop 2011; Saugstad 2011). A logical inference is that fetal programming may vary by genome.

Placental transport research involves the study of placental supply, morphology, and transfer mechanisms of gas or nutrients to the fetus (Fowden, Ward, Wooding, Forhead, & Constancia, 1996; Sibley, Glazier, & D'Souza 1997). Such research provided evidence that fetal programming may provide an explanatory mechanistic framework for the effects and consequences of maternal exposures and health status that may affect fetal development (Salafia, 2011; Sibley, 2009). Placental transport mechanisms may characterize epigenetics.

Sexual dimorphism was initially defined as qualitative descriptions of anatomical and behavioral trait differences between genders by Cunningham in 1900, but research has expanded the definitional scope to include sex-specific psychological, biochemical, gene expression, and genetic phenotypes (Gabory, Attig, & Junien, 2008; Ober, Loisel, & Gilad, 2006). Increased understanding of gender-specific metabolimics may expand the concept of sexual dimorphism.

Assumptions, Delimitations, and Limitations

The retrospective study was premised on the use of an archival genetic registry data source of recruited families with immediate family members diagnosed with autism. The AGRE registry is an ongoing collection of more than 3,308 families, not population based (Lajonchere, 2010) but expanded in 2012 by 24% to include 383 families cross-

referenced in the National Database for Autism Research federated database (Lajonchere, 2012). AGRE study results have been generalized to wider population studies of risk factors such as parental traits, maternal health, obstetric and preconception risk factors, and ASD diagnostic criteria.

The focus of AGRE is genetic research. Therefore, shared environmental exposures and family and offspring data were internally validated while family and participant identities were concealed and confidentiality preserved, which likely fostered honest responses. In addition, it is presumed the self-reported parental behavioral data were supplemental or secondary AGRE information. Therefore, Hawthorne effect bias, history, and maturation bias may be minimal. AGRE matching criteria often included familial controls that may have minimized confounding biases and reinforced the validity of shared environmental and genetic factors. Efforts were made to minimize research bias and increase study validity by using corroborating maternal, paternal, and sibling data records for parental behaviors rather than childhood biomarkers. Consideration to the temporal aspects of exposures was addressed by the conceptual framework of preconception, pregnancy, and neonatal factors (Burstyn et al., 2011; Dietert et al., 2011; Gregory et al., 2013). Another study goal was to achieve adequate statistical power to investigate the gender differential of ASD using the AGRE registry sample. Assumptions were made with regard to the level and type of matching protocols used by previous AGRE researchers. Matched controls identified and assigned within the data registry data set were presumed to be minimally biased, and any source of information bias was presumed to be nondifferential. Matching protocols were described by previous

researchers but may not be standardized among researchers (Anello et al., 2008; Cantor et al., 2007; Carayol et al., 2011; Cheslack-Pestova et al., 2007; Hallmayer et al., 2011; Serajee, Nabi, Zhong, & Huq, 2004; Stone et al., 2004; Strom et al., 2010; Wallace et al., 2008). In this study, controls reflected both familial and nonfamilial records in data analysis. A comparative analysis of familial and nonfamilial controls was conducted in this dissertation to assess the degree of participant and control group homogeneity.

Independent variables used in this study were presumed to be operationalized or reclassified with minimal bias as done by previous AGRE researchers. The tiered clinical definition of strict and broad ASD definition used with the AGRE registry has been commonly used by other U.S. researchers (Heiderken et al., 2005; Honda et al., 2009; Mayes et al., 2009; Posserud et al., 2009). Studies reconfirmed the acceptable validity and reliability of clinical ASD case definition used by AGRE researchers under proposed *DSM-V* criteria (Huerta, Bishop, Duncan, Hus, & Lord, 2012). ADIR is considered a more strict ASD criterion than ADOS (Martin & Horriat, 2012). Factor analysis for predictive ASD is commonly done using the formalized interview format used to construct ADIR scores (Norris et al., 2012).

The scope and delimitations of the study reflect the AGRE inclusion and exclusion criteria during recruitment of families (Lajonchere, 2010). However, the registry criteria of language fluency, recent birth records, standardized ASD definition, and exclusion of single-gene disorders and children with low intelligence quotient (IQ) scores were not expected to adversely affect this research. Inclusion criteria required at least one English speaking parent; and recruitment priority was given to families with

two or more immediate family members affected with ASD (Lajonchere, 2010). The AGRE registry inclusion criteria may reduce any potential impact of medical record misclassification and increase genetic homogeneity. Autism affected offspring in the AGRE were all born since 1992 (Cantor et al., 2007). Thus, risks of using disparate or changes in ASD diagnostic criteria were minimized regarding the distinction of intellectual disability (i.e., IQ less than 70) and ASD specified in ICD-9 and DSM-IV clinical criteria (Lauritsen et al., 2005).

The U.S. based AGRE databases were not relational databases with a standardized structure, which presented limitations with regard to the recoding values for particular independent variables. Data for maternal preconception diet factors were largely unavailable. Smoke exposure was presumed to reflect maternal, paternal, or household smoke exposure. For this study, prior maternal smoking behavior, maternal age, and any duration or AGRE defined dedicated lactation practice were deemed to best fit the conceptual construct in Figure 2.

Nonstandardization of matching strategies required data pooling and adjustment for missing values. Treatment of missing values involved comparing maternal and paternal survey responses, temporal assignment of risk behavior in antecedent periods, and assumptions of shared smoke and multivitamin exposures for multiple birth deliveries and siblings. History, maturation, and self-reported parental recall were study limitations of the retrospectively collected quantified variables for individuals and registry family members. Predetermined ordinal coding was proposed to minimize possible biases and test for misclassification bias.

The study outcome, presence or lack of case by clinical or medical assessment using the *DSM* or *ICD* autism diagnostic criteria, was presumed to be independent of exclusion factors. Categorical treatment of the dependent variable included the validated AGRE classification of broad ASD: ADIR positive *or* ADOS positive criteria, wherein each ASD outcome was binary coded. Outcome exclusion criteria included child or parental Fragile X, trisomy or quadrupled 15q11-13, trisomy 21 & Xp22.3, Rett or Tourette's syndrome, phenylketonuria (PKU), Tuberous sclerosis, Angelman's, Timothy Syndrome, Prader-Willis, mental retardation, and Wechsler Intelligence Scale for Children score < 70 as recorded on AGRE Child Medical History Survey Instrument. Reproducibility of results with previous AGRE researchers is presumed to suggest adequate internal and external study validity (Carayol et al., 2011; Stone et al., 2004; Yonan et al., 2003).

The focus of the AGRE registry has historically been on genetic blood sampling and genome wide association test analysis within a well-characterized familial genomic population. However, AGRE studies on the relationship of pertinent research variables to ASD diagnosis and severity have shown generalizability to other broader cross-sectional population studies. Specifically, studies on the relationship of parental age, obstetric complications, preconception health, birth order, and ASD with the AGRE registry confirmed generalized trends of other studies (Anello et al., 2009; Cantor et al., 2007; Martin & Horriat, 2012; Wallace et al., 2008). Prior use of assisted reproductive technology was asked in the AGRE surveys. The further analysis of preconception, pregnancy, and early life exposure variables in AGRE archival datasets may validate

generalized findings of other researchers and inform the metabolic and genomic findings to advance research into the pathobiology of ASD.

Significance of the Study

The relative contribution of genetic and gene-environment or epigenetic factors affecting autism diagnosis is not well understood despite decades of pathobiological research. Additional research was needed to explore the exposure-timing of risk exposures associated with ASD risk and the ASD gender differential. The gender risk differential has been accepted as a social and biological norm with little rationale provided for the implications for medical or educational systems. This study has implications for health science research, applied health literacy and educational strategies, and positive social change for individuals, families, and communities. The health science impact of the study was to add to the knowledge base of ASD etiology and the ASD gender differential. The study outcomes provided insight into the framework of Burstyn et al. (2011), who classified factors as distal, confounding, and main effect variables. The constructs of the multiliability threshold and fetal programming may be better understood given the proxy variables in this study associated with in utero exposure parameters. Study outcomes may inform the proportional risk of shared and unique exposures, preconception health, and reproductive health impact on ASD and the ASD gender-risk by exploring exposure-timing relationships. These study outcomes may impact future observational and prospective study designs given the variables identified in this study. Some study variables reflect controllable health behaviors during preconception and pregnancy with primary preventive practice implications.

Autism research continues to focus efforts toward better understanding of preconception, prenatal care, and environmental antecedents of maternal and child health. Such insights may inform preventive health strategies and refocus attention on preventive, preconception, and prenatal health planning. Surprisingly, few studies have explored the relationship prenatal maternal health and lactation to ASD risk. Maternal diet and health before and during pregnancy may be associated with lactation capacity as well as ASD risk. Insights into differences in maternal diet or lactation behavior; which may be associated with nutrient metabolism differences, may inform the ASD gender differential. Better understanding of factors affecting the gender differential risk of autism diagnosis may inform future prospective studies on autism or plausible metabolomic studies of the ASD gender differential.

Awareness of mediating factors in SIDS prevention, such as smoke exposure, fetal hypoxia, infant respiratory health, interrupted sleep or breathing patterns, and efforts to improve lactation competence among new mothers are possible implications of this study. Study outcomes may increase recognition and understanding of reproductive risk profiles associated with subsequent ASD with implications for primary prevention. If genetic, epigenetic, environmental, or prenatal health antecedents are gender-based and associated with confirmed diagnosis, preventive prenatal care and behavioral modification may be useful in reducing onset, prevalence, and management of ASD diagnosis. The gender-differentiated ASD risk may have a relationship with infant respiratory distress and family size or gravida. A better understanding of the factors that affect gender differences may inform reproductive risk profiles of women of childbearing

age. In this study, the exposure-timing risk relationships were suggestively related in meaningful ways. It is my hope the study outcomes may challenge the focus on tertiary care and management of ASD symptomology toward preventive health care.

The positive social change implications of this study have potential considering the burden of autism on health care and educational systems as well as the economic, social, and quality of life impact on communities, families, and individuals. Autism is rated the most expensive disability due to extended insurance needs, medical costs, and lack of living and employment skills (Cimera & Cowan, 2009). The educational costs of ASD are approximately 3 times higher than for traditional social, constructivist learners (Karim, 2009). ASD disrupts the social fabric of communities, schools, and families. Families with affected children have higher physical, emotional, mental, and financial stress, social and communication challenges with teachers and school staff and medical professionals, and reduced workloads and pay to caretake for their child (Woodgate, Ateah, & Secco, 2008). More than 3,70 PhD dissertations and theses related to ASD were published in ProQuest since 2001, but only 353 addressed biomedics, physiology, or etiology. Of the 52 Walden University PhD dissertations on the topic of ASD published in ProQuest since 2001, 51 were affiliated with the Schools of Psychology or Education. Only one dissertation focused on ASD etiology (Hendrix, 2011). Additional basic and applied biomedical etiology research is needed to better understand ASD risk profiles and the ASD gender risk differential.

Summary and Transition

Autism spectrum diagnosis is a life-long condition with no biological cure. The increased global prevalence and public health costs of autism are staggering. There are few theories that can explain the onset of autism, despite the presumption that biological causes are involved (Rutter, 1968). Autism genomic research suggests less than 20% of cases are directly attributed to genetic risk factors (Bukelis, Porter, Zimmerman, & Tierney, 2007; Miles, McCatheren, Strichter, & Shinawi, 2010). Despite the increased number of cases of autism, the gender differential risk is stable. The gender differential risk has consistently been reported to be approximately 4-fold higher for males (Elsabbagh et al., 2012) for standardized diagnostic criteria used by psychologists, physicians, and educational professionals (Daniels et al., 2011; DHHS, 2008). Further research into the gender differential of autism diagnosis may shed light on the mechanisms of ASD onset.

Three epigenetic pathways and six altered metabolic processes reflecting a general profile of biochemical dysfunction have been characterized for autism (Abrahams & Geschwind, 2008; Gabory et al., 2009; Hu et al., 2009). Basic research on the relative contribution and interactions of gene expression, gene-environment, and affective status or phenotypic expression of autism (Lathrop, 1993) have recently gained intense research attention (Gaita et al., 2010; Khoury et al., 2010; Meany, 2010; Tordjman et al., 2014). However, these mechanisms do not adequately account for the gender differential. Common gender-associated risk profiles have been reported for other developmental disorders and delays, sudden infant death, and nonregressive autism. ART may play a

role in these trends (Jenkins, 2013; Schieve et al., 2011), but validated prevalence estimates of the technology are still under investigation (Barradas et al., 2012). Parental preconception health-based need for ART may confound ASD risk factor contributions.

Due to uncertain etiology, indeterminate biomarker identification, and disparate timing of onset and diagnosis, ASD studies tend to be ecological and retrospective in nature. Case-control designs using state-level vital record and birth certificates are the norm. However, these records do not support preconception medical histories (Barradas et al., 2012). A focus on a hierarchical model of detailed, prioritized preconception, parental, obstetric, prenatal diet, and well documented epigenetic factors may provide a degree of study control and reflect the temporality of exposure risk factors. To this end, an exposure-model to describe temporal, interrelated risk factors for the onset of initial, nonreversible autism was recently published (Burstyn et al., 2011). The researchers used provincial infant delivery records, speculated smoke exposure was underreported, and did not account for potential use of assisted reproductive technology, instead using covariates of maternal age, maternal diabetes, and weight gain.

Improved awareness of the relative contribution of indirect and direct pathobiological proxy variables for genetic, maternal, and epigenetic biomarkers and infant/childhood trait risk profiles may inform plausible, gender-specific risk profiles for subsequent autism diagnosis. A better understanding of the possible relationship of plausible gender-specific, biochemical risk factors and clinically confirmed ASD diagnosis may inform possible biomedical mechanisms, facilitate prevention and medical intervention, and support family and individual behavioral change.

A pragmatic, retrospective study of the relationship of genetic inheritance, prenatal, and neonatal exposure-timing of parental and infant risk factors may inform exposure modeling and provide insight into primary prevention strategies for ASD. There are advantages to design studies using a narrowed focus on preconception health (with control of ART impact), prenatal maternal health, and early life conditions of captively controlled infant dietary intake. These specific factors may reflect plausible fetal programming and placental transfer mechanisms.

A case-control study, with temporal independent exposure-dose considerations for the hypothesized relationship of pregnancy related main effect variables of maternal smoke exposure via self, partner, paternal, or household smoke exposure, maternal diet, lactation, and gender on ASD was needed. As a major premise, pregnancy risk factors were presumed to be confounded by neonatal traits such as infant respiratory status, as measured by regularity of infant breathing during waking hours and infant sleep patterns. The impact of hypothesized covariates of the preconception period on the exposure-timing relationship to ASD was an additional major premise in this thesis. Distal antecedent factors of parental age and preconception maternal health status may mediate epigenetic mechanisms associated with maternal health status during pregnancy. Obstetric and delivery complications were theorized in this study to reflect preexisting preconception health or other underlying genetic susceptibility risk factors.

The effect modification of gender on association of these exposure-timing variables to autism diagnosis is also of interest since substantial antidotal evidence exists for the gender differential of ASD, with little biological explanation. Therefore, fetal

gender was presumed to be a main effect variable in the conceptual framework and interpreted in light of the independent adjustment for confounders and distal correlates of the main effect relationships. Child gender is also a possible confounder in the relationship of pregnancy factors and ASD outcome. Neonatal proxy variables were hypothesized to be associated with extraneous factors such as respiratory infections, side effects of psychotherapy treatment, congenital heart defects are presumed to confound the hypothesized main effect relationship of pregnancy factors and ASD.

The logic and rationale for the temporal clustering of the study variables into preconception-distal factors, fetal exposures during pregnancy, and lactation as main effect variables, and confounding infant traits was based on the state of the scientific literature as detailed in Chapter 2. The effect estimate for the covariation of pregnancy variables with ASD outcome and rationale for temporal precedence and the hierarchical exposure-timing hypotheses are summarized as part of the literature review in Chapter 2.

Chapter 2: Literature Review

Introduction

The purpose of this quantitative study was to focus on early life and prenatal factors to describe the relationship between parental and childhood traits or biomarkers and gender-stratified analysis of autism diagnosis. The purpose of this chapter is to draw parallels among risk factors of other developmental disorders, sudden infant death, and autism and highlight plausible pathways and biomarkers reported in autism etiology research. In this chapter, I include a review of ecological trends in autism and developmental disorders, diagnostic criteria for autism, and a literature review of biochemical pathways, critical windows of exposure associated with autism risk factors as well as a discussion of the challenges of identifying autism biomarkers. Suggestive and reproducible evidence in the literature for plausible relationships between risk factors of preconception parental age, smoking status, obstetric complications, and pregnancy factors such as maternal smoking, perinatal diet, lactation, and the gender differential of autism are summarized in the final section of this literature review.

Fewer than 10 papers have described gender-based symptomology of autism (Klusek, Losh, & Martin, 2014; Rivet & Matson, 2011). While generalized pathways of metabolic dysfunction associated with autism diagnosis have been validated by many independent researchers, less than 15 papers were identified in this literature review that described gender-based metabolic or dietary biomarkers among persons healthy persons stratified by gender or for group comparisons of children with autism and matched controls. Literature related to genetic, hormonal, and environmental factors affecting

metabolic gender-differential of unaffected children were included in the review due to the lack of studies specifically focused on male predominance risk of clinical initial autism diagnosis at age 8.

Literature Search Strategy

The purpose of this review is to provide background information on replicated studies that identified medical or biomedical risk factors associated with diagnosis on the autism spectrum. ASD case definition criteria were reviewed, as were biological pathways and candidate biomarkers associated with diagnosis. The review compared and contrasted biological plausibility and gender stratified results for risk factors among parents and offspring. A review of the etiologic aspects of the ASD research literature focused on maternal or parental genetic, prenatal, environmental, or behavioral factors associated with subsequent clinical diagnosis. The literature scope was limited to articles published in the English language since 1960, with predominantly U. S. based sampling frames or datasets represented by U.S. cohorts and maintained by American researchers. The rationale for including literature published since 1960 was to make sure that articles on infant newborn screening initiatives in the United States were captured as well as special education literature publications and the emergence of autism etiology research (Newschaffer et al., 2012).

The following databases were used: ABI/INFORM Global, Academic Complete Search Premier, Cochrane Database of Systematic Reviews, EBSCO, Health and Social Care Encyclopedias from Sage, Education Research Complete, Expanded Academic ASAP, Google Scholar, Health and Medical Complete (ProQuest), PubMed, Health

Sciences: A Sage Full-Text Collection, InfoSci Journal, MEDLINE with Full Text, ProQuest Central, and Thoreau. The following keywords were used: *autism, epidemiology, autism spectrum disorder, case definition, diagnostic criteria, behavioral and environmental risk factors, etiology, biologic mechanisms, pathways, risk factors, exposure, maternal health, paternal health, parental age, smoking, maternal diet, nutrition, protein intake, fatty acids, lipids, obstetric complications, assisted reproductive technology, biomarkers, genetics, genome wide association studies, immune, gut health, epigenetic, imprinting, environmental exposure, environmental risk; exposures; prenatal; gender-differential; sexual dimorphism; gender; sex; metabolism, prevalence, sudden infant death syndrome, placental transfer, and metabolomics*. Only text in English was reviewed; the most relevant literature has been published since 2000. Articles from the reference lists of previously obtained articles were included. Gender-stratified analysis and the gender-differential literature were noted to be more frequent after theories of “excessive male brain” and androgen literature were published (Baron-Cohen, 2002; Mills et al., 2007).

The literature search strategy focus for the outcome variable was nonregressive autism spectrum diagnosis with exclusion of specific congenital gene mutations such as Rett’s, Prader-Willis, Angelman’s syndrome, Fragile X, phenylketonuria, and tuberous sclerosis. The search strategy included a focus on gender comparison study designs for independent variables of parental behavioral traits, infant traits, neurotransmitter, and endocrine and hormonal biomarkers associated with autism spectrum disorders in peer reviewed scholarly journals. Generalized findings of gender-differentiated serum

metabolites among healthy adults and gender-associated autism diagnosis symptomology were summarized by Ober et al. (2008) and Rivet and Matson (2011), respectively.

The scope and purpose of this study was based on biomedical pathway and marker research rather than psychosocial dimensions of autism. Research papers on comparative anatomy based on postmortem, tomography, or magnetic resonance imaging analysis of brain anatomy and brain region physiology among cases and controls were not a focus of the literature review. The scope of the literature review was observational studies of prenatal and neonatal biomarkers and developmental risk profiles among childhood populations. Original research articles based on anatomical gender-differences in human or rodent models published in English after 2008 were included if the study focus was neonatal or neurochemical antecedent biomarkers, gene-expression, epigenetic methylation, or neurotransmitter metabolites known to affect neonatal brain region development.

More than 50 genome wide association research studies have been published since 2001 on autistic patients and matched controls but were not a focus of the behavioral traits literature review, and few reported gender differentials among cases (Miles, McCathren, Stichter, & Shinawi, 2010; Yu et al., 2013; Yuan & Dougherty, 2014). Emerging studies suggested maternal allele variations, phenotypic expression, and neurosteroid influence during fetal brain development may be antecedent risk factors of autism (Courchesne, 1997; Ebstein et al., 2009; Fukumoto et al., 2009; Habek & Kovacevic, 2011; Lerer et al., 2008; Redclay & Courchesne, 2005; Scheiffele, 2011; Yuan & Dougherty, 2014). Geocultural, haploid, and phenotype variance and blood

relation diversity impact gene allele pools across race/ethnicity and region (Gaita et al., 2010; Khoury et al., 2010; Meaney, 2010). Sex-specific genetic variation has been documented.

Suggestive and reproducible evidence in the literature for plausible relationships between independent risk factors of preconception parental age, smoking status, obstetric complications, and pregnancy factors such as maternal smoking, perinatal diet, lactation, and the gender differential of autism are summarized in the final section of this literature review. A review of published research on the effect of parental and infant variables used in this study for the intended study cohort, AGRE, a well recognized open access autism registry, is also described. The AGRE population was the sampling frame for this study. Thus, observational study designs for AGRE cohorts were also researched and included in this literature review. A summary of AGRE population genetic characterization studies stratified by infant gender was included in the final section of this literature review.

Initial Autism Clinical Case Definition

There is high agreement and definitional overlap between *DSM-IV* and ICD-10 diagnostic case criteria for initial autism diagnosis (see Appendix A); both describe social dysfunction in at least three domains. Cognitive and psychological constructs for autism (*DSM-IV*) are valid and reliable (Heiderken et al., 2005; Honda et al., 2009; Kim et al., 2014; Mayes et al., 2009; Posserud et al., 2009). The U.S. Department of Education added supplemental criteria to the *DSM-IV* and ICD-10 autism case criteria, and diagnosis is typically done at 8 years of age. U.S. public schools have added criteria for nonperformance at grade level as an ASD diagnostic metric (CFR, 2008). It should be

noted that the current CDC surveillance program (CDC, 2007a, CDC 2007b, 2009, 2012) is based on the Code of Federal Regulations (2008) case definition of autism spectrum disorders. Because of the concern over lack of possible nonstandardized assessment of these additional criteria, the literature review was limited to case definitions based on *DSM-III*, *DSM-IV*, *DSM-IV-TR*, and/or ICD criteria, rather than Code of Federal Regulation case definitions.

Biological Pathways Implicated in Autism

There were numerous articles identified that described hypothetical, biological pathway dysfunction associated with autism diagnosis. Three seminal papers independently detailed epigenetic mechanisms and potential metabolic consequences of those associated mechanisms (Abrahams & Geschwind, 2008; Gabory et al., 2009; Hu et al., 2009). Gabory et al. (2009) proposed that multigenerational epigenetic mechanisms associated with nuclear receptor sites, direct chromatin modification, and membrane-receptor signalling cascades were triggered in pregnant woman in response to environmental exposures such a dietary intake, pharmacotherapy, or chemical exposure. Hu et al. (2009) studied the genome wide association differences using a pseudo-case control design for ASD children and nonaffected siblings enrolled in the AGRE cohort to compare genomic profile by ASD and gender. They concluded genes implicated in nervous system development, inflammation, and cytoskeletal organization were associated with ASD. Cholesterol/steroid metabolism and androgenic hormones were speculated to be associated with the gender differential of ASD. Moreover, Tordjman et al. (2014) reviewed a multitude of plausible epigenetic remodeling mechanisms

associated with ASD. The study of documented prenatal exposures and epigenetic factors using a well-characterized genetic cohort may provide insight into the possible role of epigenetic alterations in ASD.

Abrahams and Geschwind (2008) summarized autism disorders as involving inhibitory synapse mechanisms, serotonin, glutamatergic, and/or calcium signaling pathway components among cases and controls. These molecular pathways were consistently proposed in the published literature to hypothesize etiology of diagnosis but the results were rarely, if ever, discussed with regard to a rationale for the ASD gender-differential (Adams et al., 2011; Erickson et al., 2005; Evans et al., 2008; McGinnis, 2004; Schultz et al., 2008). The mechanism-related literature results did not propose biomarkers or proxy variables for the proposed pathways and lacked evidence of unifying biological plausibility, timing, specificity of mechanism, or exposure and biological gradient to address attributable risk by gender. Reviews of the limitations of identifying valid, reliable, and specific biomarker candidates for the generalized six pathways was recently published (Ratajczak, 2011; Tordjman et al., 2014), but the discernment of biomarkers that may inform the ASD gender-differential was lacking.

The epigenetic mechanisms described by Gabory et al. (2009) associated genetic profile clusters suggested by Hu et al. (2009) and metabolic pathway consequences detailed by Abrahams and Geschwind (2008) and could be hypothetically unified in an exposure-effect model of maternal dietary factors associated with autism. Maternal health status certainly affects fetal health risks including ASD risk (Saugstad, 2011). Cultural trends toward reduced milk and dairy consumption and reduced sunlight

exposure have been speculated to be coincidental with an increase in autism prevalence (Cannell, 2008; Grant & Sales, 2009). Research has indicated maternal and fetal genetic and nutritional status of Vitamin D and related binding proteins may be important gender level predictors of autism risk (Cannell, 2008; Cannell & Hollis, 2008; Grant & Sales, 2009; McGrath et al., 2001) and explain the gender-risk differential (Bolland et al., 2007; Hagenau et al., 2009).

However, it is unclear from the literature whether these biomarkers reflect phenotypic symptomology, coincidence with offspring dietary patterns, underlying gender metabolism or hormonal differences in biochemical regulation, comorbidity, or underlying autism etiology mechanisms. Biomarkers have not been identified as reliable, independent, or main-effect indicators of gender differences in autism onset, progress, or mediation. The relationship of prenatal maternal health status and autism diagnosis in offspring is not well understood and deserves more study. However, the hypothesis that epigenetic factors appear to be associated with subsequent autism diagnosis is well documented. Research on the dose, type, and timing of exposures associated with epigenetics, adverse fetal development, and subsequent autism risk deserves additional research attention. Such research may inform the gender differential in ASD.

Critical Windows of Exposure Associated With Autism

Autism research continues to focus efforts toward better understanding of prenatal care and environmental antecedents of maternal and child health. The study of genetic susceptibility, gene-environment interactions, and perinatal health status on ASD and the gender differential of ASD require study frameworks that can account for

exposure variables before conception as well as gene-environmental interactions before conception and during pregnancy and delivery.

Dietert et al. (2011) summarized putative critical windows of prenatal environmental exposures for autism risk by pregnancy trimester as illustrated in Table 1 based on evidence from rodent and human studies.

First trimester exposure to valproic acid (contained in antiepileptic medicine), thalidomide, misoprostal, lack of food aversion and vomiting, viral infection, and hospitalization were cited as risk factors for ASD (Dietert et al., 2011). Valproic acid has been shown to potentiate the response of inflammatory factors and interleukins in mice (Awale, 2012). A reduction in macrophage activation occurred in both male and female mice but was more pronounced in male mice and reproduced using two separate macrophage cell lines.

Table 1

Prenatal Exposures Associated With Subsequent Autism Diagnosis

Prenatal trimester	Exposure risk factor for subsequent autism diagnosis
First	Valproic acid, thalidomide, misoprostal Food aversion and maternal vomiting Viral infections
Second	Bacterial and/or murine viral infections Maternal psychosocial stress and/or depression
Third	Vitamin D deficiency, terbutaline Chorioamnionitis infection Premature birth and/or low birth weight

Habek and Kovacevic (2011) reported fetal hypoxia verified by ultrasonography at 10 to 20 weeks gestation in pregnant women who smoked more than 20 cigarettes

daily. The authors followed the course of pregnancy of 60 women (grouped by smoking behavior) from gestation to offspring health outcomes at age 10 and reported increased broncho-obstructive syndrome, fetal hypoxia, and SIDS risk increased for mothers who smoked more than 10 cigarettes per day.

Other researchers reported bacterial or murine viral infections and general maternal stress were risk factors in the second trimester of pregnancy (Dietert et al., 2011). Using pregnant mice exposed to *influenza A* virus, Miller et al. (2013) reported exposure in first trimester produced behavioral symptoms such as loss of locomotor control particularly in male offspring. The authors speculated sex-specific dopamine or brainstem inflammation was significantly higher in male offspring based on tissue assays (Miller et al., 2013). Bacterial metabolites, such as 3-hydroxyphenylalanine have also been shown to affect brain catecholamine levels and be associated with ASD (Shaw, 2010). Some research has shown a tendency toward higher male-associated maternal transmission or placental transfer risk of measles and respiratory syncytial viral infections (i.e., a risk factor for SIDS); but a recent published meta-analysis concluded little increased gender risk of ASD due to prenatal, neonatal, or childhood infections (Zerbo et al., 2013). However, maternal fever during pregnancy was associated with ASD and pervasive developmental disabilities $OR= 2.12$, 95% CI [1.17 - 3.84]. Cannell (2014) proposed the use of paracetamol, fever reducing chemical found in brands such as Anacin®, DayQuil® and Tylenol® increased oxidative stress, particularly in vitamin-D deficient pregnant women and may be a contributing factor to ASD.

Vitamin D deficiency and use of terbutaline or pitocin, a treatment used to delay premature birth were cited as environmental risk factors in the third trimester of pregnancy by Dietert et al. (2011). Low maternal vitamin D status (< 75 nmol/l at 32 weeks gestation) was shown to be associated with reduced developmental language scores at age 6 months compared to children born to rural Vietnamese women with higher 25-hydroxyvitamin D levels (Hanieh et al., 2014). Low prenatal vitamin D levels (25-OHD) were also associated with small for gestational age, larger head circumference at birth, and lower infant length-for-age at six months ($n=960$). Neggers (2014) reviewed several theories which have emerged to explain the relationship of periconception and maternal vitamin D deficiency and ASD risk in offspring which include increased cellular oxidation, reduced anti-inflammatory potential, hormonal or enzyme activation disruption, and DNA repair and maintenance.

Periconception vitamin use has been associated with reduced risk of offspring ASD. Braun et al., (2014b) reported second trimester prenatal vitamin use and maternal whole blood folate levels were positively associated with social responsiveness scales for children aged 4-5 years among 209 mother-child pairs. Schmidt, Tancredi, Krakowiak, Hansen, and Ozonoff (2014) reported higher maternal iron intake from dietary supplements and cereals periconceptionally, during pregnancy and lactation was associated with reduced ASD risk. Mothers of cases were less likely to report using iron supplements and had lower mean daily iron intake estimates. Schmidt et al., (2012) studied the relationship of prenatal vitamin use on genetic alleles associated with methylation, and the risk of ASD in a U.S. population-based case-control study. Results

showed prenatal folic acid use three months before pregnancy, or the first month of pregnancy was associated with lower ASD risk. Risk of ASD was highest for mothers who did not take vitamins periconceptionally. There was a significant interaction with regard to genetic allele form of catechol-O-methyltransferase (COMT), tetrahydrofolate reductase, (MTHFR) and CBS (enzyme cofactor for Vitamin B6) genes associated with vitamin B metabolism. ASD risk was greater for particular maternal MTHFR and CBS, and infant COMT-472AA genetic alleles. COMT codes for an enzyme associated with dopamine, adrenaline and noradrenaline regulation, and has been associated with other development disabilities such as ADHD (Matthews et al., 2012; Palmason et al., 2010; Vorstman et al., 2009). Saugstad (2011) proposed lack of prenatal essential fatty acids in the third trimester affected epigenetics associated with SIDS risk. The effect of exposure by trimester may be associated with particular timing of fetal developmental milestones. Pineal gland expression is known to occur within three weeks after conception in rodents (Munoz, et al., 2007) and three human primary brain vesicles are evident by four weeks (Marieb, 2001a). Immune development, triggered by tissue and organ seeding begins at four to seven weeks gestation (Leibnitz, 2005) concurrent with brain microglia cell differentiation (Monier, Evrard, Gressens, & Verney 2006) while thymic events are thought occur 8-18 weeks gestation (Leibnitz, 2005). Myelination of fetal nerve cells begins around 24-25 weeks gestation and accelerates to a peak growth rate at age one year (Steinman & Mankuta, 2013). Women carrying female fetuses have significantly higher placental growth hormone in their blood than women carrying male fetuses at 28 weeks gestation; which may be a key factor in subsequent offspring ASD (Steinman &

Mankuta, 2013). Little brain synaptic development occurs before the third trimester of pregnancy; at which time the rate accelerates to 40,000 synaptic connections per minute and continues to childhood calendar age of two years (Bourgeois, 1997). There is a strong relationship between synaptic connection rate and glucose metabolism rate (Johnson, 2003). Gender may play a role in glucose metabolism based on studies in healthy adults and children with autism (Evans et al., 2008; Mittelstrass et al., 2011).

Cellular dysfunction, mitochondrial distress, lack of homeostatic control, and exogenous stress associated with early or initial autism diagnosis provides evidence for the hypothesis that autism onset or antecedent trigger mechanisms for risks of autism onset likely occur within perinatal and prenatal periods. Prenatal exposure evidence for the gender differential of ASD is not well understood, has not been extensively studied, and deserves further investigation.

Gene-environment interaction, more specifically, maternal gene-diet/exposure factors may be related to fetal programming and subsequent onset risks of autism. Gabory et al., (2009) described three broad types of epigenetic mechanisms leading to sexual dimorphism of second to fifth generation (F2-F5) offspring :

1. nuclear receptor binders such as endocrine disruptors, genestein, bisphenol A, retinol, peroxisome, polyunsaturated fatty acids, drug fibrates which may alter chromatin remodeling enzyme(s) function(s);
2. direct chromatin modification via folate deficiency, methionine, resveratrol, sulphoraphane, valproate (valproic acid exposure has been associated with the onset of autism-like traits in rats), trichostatine A, fungicide/vinclozolin, and

corticoid steroids/dexamethasone exposure;

3. membrane-receptor signalling cascades due to social/behavioral responses affecting serotonin, cortisol cascades, and sodium, potassium, and calcium signalling channels.

Many theorized autism pathway mechanisms rely on animal-based (e.g. rodent) studies with fewer published human studies (Dietert et al., 2011; Tordjman et al., 2014). Rogers cautioned that rodents are born developmentally earlier than humans, thus third trimester human development mechanisms are postnatal mechanisms in rodent models (Brown, Sawyer, & Grossblatt, 2011). Heindel agreed and noted animal models are not typically used for longitudinal or later-onset disease research stage (Brown et al., 2011). Researchers have cited the common use of inbred strains of laboratory animals reduces the ability to study gene-environment interactions (Brown et al., 2011; Shelton, Hertz-Picciotto, & Pessah, 2012). Thus, the validity of rodent models to predict human fetal exposure-effect relationships associated with autism etiology is questionable. However, the ability to study prenatal risk factors and the gender differential is likely more problematic in humans.

With regard to human population studies, cultural factors, longitudinal changes in maternal behaviors, obstetric and medical practices, and subtle refinements in case definition diagnosis complicate estimation of autism incidence. From an ecological perspective, McDonald and Paul (2010) used literature reviews from three longitudinal studies (~10 years) to determine cumulative incidence of autism within each cohort study for children diagnosed at age five years or older. In Danish, California, and Japanese

cohorts for the period 1988-1996, cumulative incidence for all five core subtypes of autism increased; 1988-1989 was identified as an inflection point representing accelerated prevalence rates for the Danish and California cohort studies whereas the Japanese studied showed a constant incidence rate increase. In the Danish cohort, acknowledgement of ICD-8 case definition was used prior to adoption of ICD-10 case definition in 1993 (Lauritsen, Pedersen, & Mortensen, 2005). Therefore, the seminal distinctions of childhood schizophrenia, mental retardation, and infantile autism defined in ICD-9 criteria may not have been used in the Danish cohort analysis using case counts prior to 1993. In addition, the changepoint years of 1988-1993 were also coincidental with the increased popularity of assisted reproductive technology (Schieve et al., 2011). Germ cell imprinting, a key concern for assisted reproductions has been documented (Halliday, 2007) and associated with lower birth weight, preterm birth (McDonald, Han, Mulla, Murphy, Beyene, & Ohlsson, 2009) obstetric complications, multiple births (Halliday, 2007; Schieve, et al., 2011) and risk of autism diagnosis in the U.S., Finland, and Japan (Klemetti, Sevon, Gissler, & Hemminki, 2006; Schieve et al., 2011; Shimada et al., 2012). These risk factors tended to more adversely affect boys more so than girls. Sandin, Nygren, Iliadou, Hultman, and Reichenberg (2013) reported the specific technique of intracytoplasmic spermatozoid injection was associated with higher ASD.

Therefore, there was suggestive evidence that common and specific prenatal exposures during pregnancy affect subsequent autism diagnosis based on rodent models and ecological human studies. These exposures may be associated with epigenetic changes which may be associated with post-natal metabolic and nutritional biomarker

profiles. It was of interest whether gender-differences in infant proxy biomarker profiles or arrays were reported in the literature.

Inconclusive Childhood Biomarker Candidates for Autism

Ratajczak (2011) recently reviewed key biomarker candidates of immunology, gastrointestinal function, neurologic and toxicological systems associated with autism published in the Pub-Med and Ovid Medline database literature since 1943 and proposed the hypothetical concept of a comprehensive biomarker array profile with attributed autism risk contributions from risk factors. Suggestive evidence of specific epigenetic or metabolic pathways such as those described above were hypothesized by Ratajczak (2011) and Wilson (2014) to explain the association of risk factors and subsequent diagnosis of autism. It would seem valid and reliable biomarker candidates or biochemical intermediates must serve as proxy variables for the proposed metabolic pathways. It was of interest whether these proxy biomarkers may also inform the gender risk differential in autism. The literature regarding biomarker feasibility of interleukin, cytokines, immunoglobulins, hormones and catecholamines as confirmatory proxy biochemical intermediates for ASD and the gender differential in ASD have been summarized and showed mixed results (Corbett et al., 2008; Geier et al., 2009; Kern et al., 2011; Rosen, Yoshida & Croen, 2007; Steinman & Mankuta, 2013). The relationship of ASD and interleukins associated with maternal and infant or childhood dietary exposure have been inconclusive (Goines et al., 2011; Onore et al., 2009). Unfortunately, very few studies addressed the ASD gender differential of these biomarkers.

Stigler, Sweeten, Posey, and McDougle (2009) concluded that while a 1964 rubella epidemic was a unique, specific hazard exposure for increased risk of autism, other infectious, autoimmune, and cytokine-related etiologic studies showed mixed results. Childhood infection patterns suggested a slightly higher risk of infection within the first 30 days of life for persons with autism in a case-control study, but the trend was not evident over 2 years of the child's life (Rosen et al., 2007). In general, innate or acquired adaptive immunity responses are triggered by foreign or adverse biochemical agents, which in turn initiate a cascade of cellular responses and cytokine release across the blood-brain barrier, resulting in inflammation, fever and tissue swelling. Clearance of measles and other viruses requires the development of adaptive immunity (Marieb, 2001b; Nelson & Williams, 2007). Neonate and infant immune challenge or autoimmune disorders may lead to encephalopathy which is often coincidental with post-mortem analysis of brain matter for persons with autism (Campbell et al., 2009; McMillin, Richards, Mein, & Nelson 1999; Mills et al., 2007; Newschaffer et al., 2007). The amygdala brain has been reported to be enlarged in autistic children (Miller et al., 2013; Schumann, Barnes, Lord, & Courchesne 2009; Steyaert & Marche, 2008; Wakefield, Puleston, Montgomery, Anthony, O'Leary, & Murch 2002).

Encephalitis type physiological outcomes in infants from 1-2 years of age have been consistently associated with subsequent onset of ASD but causal mechanisms, and reliable biomarkers indicating timing or sequence of biologic events, are unknown (Corbett et al., 2008; Ghaziuddin, Zaccagnini, & Elardo, 1999; Lopata, Volker, Putnam, Thomeer, & Nida, 2008; Naber et al., 2007; Redclay & Courchesne, 2005).

Neuroanatomical abnormalities such as larger head circumference among autistic infants and toddlers (Barthomoleusz, Courchesne, & Karns, 2002; Redclay & Courchesne, 2005), enlarged grey and white matter volume in prefrontal cortex and other brain regions tissue, (Herbert et al., 2004; Polleux & Lauder, 2004) and decreased number of Purkinje cells in cerebellum tissue (Bauman & Kemper, 2005; Courchesne, 1997; Fatemi et al., 2002; Kemper & Bauman, 2002) are associated with autistic children compared to matched controls. Some researchers suggested disruption of brain cerebellar tissue development or interconnectivity, synaptic pruning, gestational insult, or limbic system imbalance were biochemical mechanisms of ASD onset (Courchesne, 1997; Kidd, 2010; Saugstad, 2011; Schumann et al., 2009).

The identification, measurement, and utility of biomarkers as proxy variables for possible autism mechanistic pathways remain a daunting epidemiologic challenge. The complex, integrated biochemical pathways, and uncertain assessment of symptomatic, coincidental, and/or co morbid conditions of human physiology result in at best, inconclusive, tentative relationships between autism diagnosis and endocrine biomarker candidates. The relationship of valid, reproducible biomarker candidates and ASD onset or the timing of autism “trigger” mechanisms deserves further study. The effect of gender on pre-conception, pregnancy and postnatal developmental trajectory biomarkers is also of interest. In addition to endocrine biomarker research, research into gender effects of dietary metabolic markers has been reported. Characterization of dietary metabolites for perinatal, fetal and neonatal exposure may inform the criticality of

epigenetic mechanisms associated with ASD. Dietary metabolic research may provide evidence of the relationship of dietary factors to the gender differential of ASD.

Childhood Dietary Metabolites as Proxy Biomarkers for Autism

The impact of compromised immune function in autistic children may present as clinical evidence of nutritional deficiencies and nutrient imbalances, but it is difficult to determine the timing of the metabolic assault. Therefore it is difficult to detail whether nutritional deficiencies were a risk factor at birth due to prenatal epigenetic factors (Braun et al., 2014b; Gabory et al., 2009; Schmidt, Tancredi, Krakowiak, Hansen, & Ozonoff, 2014) or metabolic consequences of childhood dietary inadequacies as described by Abrahams and Geschwind (2008) and others (James et al., 2010; Taurines et al., 2010). To gain insight into this phenomena, a literature review of dietary metabolites associated with autism was conducted. There were a few papers which identified dietary recall differences between autistic children and normally developing children, but studies generally did not include biomarker data and reported conflicting results (Herndon, DiGuiseppi, Johnson, Leiferman, & Reynolds 2009). These studies will be detailed in this section and include results for gender-stratified studies.

An emerging area of research in childhood autism etiology is the study of the central nervous system integration as it affects regulation and coordination of body functions under the control of limbic-hypothalamic-pituitary-adrenocortical axis (Kahn, 2012; Kidd, 2010). The relationship between gut, brain, nutritional, and toxic exposure-effects has been associated with increased cellular oxidative stress and ASD (Bradstreet, Smith, Baral, & Rossignol, 2010; Cannell, 2014; James et al., 2004; Lillycrop, 2011;

McGinnis 2004; Neggers 2014; Tordjman et al., 2014). Erickson, Stigler, Corkins, Posey, Fitzgerald, and McDougle (2005) completed a meta-analysis of metabolic and gastrointestinal symptoms suggestively associated with autism spectrum diagnosis. Several authors suggested a decreased metabolic sulfation capacity or transsulfuration abnormalities in children with autism (Geier et al., 2009; Moss & Waring, 2003). These studies and original research related to oxidative stress and sulfation metabolites, blood and urinary amino acid, vitamin D and mineral nutritional metabolites among children with autism (with or without the use of pharmacotherapy) are summarized below. A review of the study findings also illustrates the fact that few gender-specific dietary metabolite research findings were identified in the literature. Early published ecological studies emerged on diet factors; investigative environmental chemical exposure studies soon followed.

Dufault, Schnoll, Lukiw, LeBlance, and Cornett (2009) conducted a macro-ecological U.S. study and proposed cultural diet trends toward lower essential fatty acid intake, higher fish consumption and high fructose corn syrup intake affected mineral balance (i.e. higher mercury, lower zinc status), glutathione metabolism and increased oxidative cellular stress risk factors among children. These trends were suggested to increase autism risk among U.S. children. A follow-up comparative ecologic study of U.S. and Italian children suggested high fructose corn syrup and organopesticide exposures may be risk factors for onset autism prevalence among U.S. children (Dufault, Lukiw, Crider, Schnoll, Wallinga, & Deth, 2012). Dufault et al. (2009, 2012) did not report gender stratified data in either the U.S. or Italian cohorts used in the two studies.

Kinney, Barch, Chayka, Napoleon, and Munir (2010) identified nine chemical mutagenic risk exposures and four environmental factors (urbanization, geographic latitude, precipitation and sun exposure) from the literature which were speculated to be associated with ASD in the U.S..

U.S. environmental exposures to pollutants, endocrine-disrupting chemicals, occupational exposures, and agricultural pesticides have been studied and many studies reported suggestive evidence related to ASD risk (Braun et al., 2014a; Chen et al., 2014; Roberts et al., 2013; Shelton et al., 2012; Windham et al., 2013). Maternal exposure to occupational hazards such as volatile organic compounds (Windham et al.); heavy metals, petroleum-based solvents (Roberts et al.) and maternal exposure during pregnancy to polybrominated diphenyl ethers (Chen et al.) were associated with increased ASD and risk of child developmental delays for ecological studies using exposure modeling estimates. In the Nurses' Health Study II cohort, Roberts et al. reported linear, positive trends for exposure to air pollutants and risk of ASD with significantly stronger associations for boys than girls. Suggested explanations for the gender-differential were lower neurotoxic or inflammatory thresholds for boys, or sex-specific social behavioral effects related to dopamine regulation (Roberts et al.). Other researchers suggested children diagnosed with ASD have impaired detoxification mechanisms which inhibit the body's ability to adapt to increased environmental neurotoxins; regardless of child gender (Alabdali, Al-Ayadhi, & El-Ansary, 2014; DeSoto 2009). Volk, Kerin, Lurmann, Hertz-Picciotto, McConnell and Campbell (2014) reported particular allele forms of the MET

gene promoter were associated with roadway air pollution (benzopyrene) and ASD in mouse studies modeled to simulate California roadway pollution.

Other researchers reported an association of in utero levels of maternal organopesticide xenobiotic clearance as measured by higher liver enzyme paraoxonase (PON1) levels and reduced neurodevelopmental risk within a California infant birth cohort; no offspring gender differential was reported (Eskenazi et al., 2010). However, higher PON1 levels have been found to be higher in female rats and hamsters as compared to males (bin Ali, Zhang, Lim, Fang, Retnam, & Lim, 2003; Thomas-Moya, Gianotti, Llado, & Proenza, 2006). Rodent PON1 associated enzyme levels in liver tissue were equally reduced in both genders upon xenobiotic exposure (Feingold, Memon, Moser, & Grunfeld, 1998) or calorie restriction (Thomas-Moya et al., 2006). Therefore, the literature suggests effects of xenobiotic exposure and measurable detoxification mechanisms of organopesticides, volatile organic chemicals, heavy metals, high fructose corn syrup, and fatty acid intake on PON1 activity and dietary metabolite markers are inconclusive.

Early studies on autism etiology were focused on dietary biomarkers for ASD risk. Arnold, Hyman, Mooney, and Kirby (2003) analyzed plasma amino acid levels among 36 children with autism, stratified by dietary intake and matched by age and gender. Autistic children on casein and gluten restricted diets had lower plasma levels of tryptophan and tyrosine; which are neurotransmitter precursors and were speculated to reflect dietary intake. No gender-stratified analysis was reported. The authors speculated casein and gluten restricted diets may reduce serum levels of tryptophan, a serotonin

precursor, and tyrosine in autistic children. Plasma tryptophan is important for normal sleep cycle function; and coincidentally, lack of regular sleep pattern is common among children with autism (Krakowiak, Goodlin-Jones, Hertz-Picciotto, Croen, & Hansen, 2007; Margoob & Mushtaq, 2011). It is difficult to ascertain the effect of restricted diets on sleep patterns in children with autism. Dietary restriction related to opiate peptides found in dairy products is a common intervention for children with autism. The effect of pre and post-natal vitamin D associated metabolites on subsequent ASD risk is unclear.

Molloy, Kalkwarf, Manning-Courtney, Mills, and Hediger (2010) studied plasma vitamin D levels, as measured by 25 hydroxyvitamin D in male children aged 4-8 years diagnosed with autism and matched male controls. Plasma vitamin D levels were compared for unrestricted diet groups (40 cases and 40 controls) and a small subgroup of cases on casein-restricted diets ($n = 9$) after adjustment for seasonality/sunlight effect on vitamin D and the use of dietary supplements. There were no significant group differences but 61% of children had low (less than 20ng/ml) plasma vitamin D levels. This minimum level is recommended to ensure adequate bone health.

Gong et al. (2014) reported Chinese children initially enrolled in a hospital neurology department for ASD therapy had low indigenous levels of serum 25-OH vitamin D even after adjustment for age, sex, body mass index and serum levels of calcium, phosphate, magnesium and seasonality/light exposure. Serum 25-OH vitamin D levels were an independent predictor biomarker of ASD. Odds risk of low (< 20 ng/l) serum vitamin D among ASD children was 1.23, 95% CI [1.10-1.37]. Xia, Zhou, Sun, Wang, and Wu (2013) showed that while severe malnutrition was a factor in 8.1% of

children enrolled in a ASD clinic, dietary recall methods shows that 111 children aged 2-9 had low daily intakes of vitamin A, B6, C, folic acid, calcium and zinc. However, vitamin E, niacin, iron and magnesium intake exceeded 80% of daily recommended intakes and growth rates were typical for the cohort of Chinese children.

Other researchers also investigated the relationship of childhood plasma vitamin D levels and autism diagnosis, but studies did not control for diet, sunlight or dietary practices and/or showed no differences among cases and controls (Fernell, Barnevik-Olsson, Bagenholm, Gillberg, Gustafsson, & Saaf, 2010; Grant & Sales, 2009; Meguid, Hashish, Anwar, & Sidhom, 2010). Other researchers reported dairy mineral levels were associated with ASD (Adams et al., 2011). Sulfur metabolism biomarkers have been suggested to differ among cases and controls.

Adams et al. (2011) reported lower plasma glutathione, sulfate, tryptophan and higher levels of oxidative stress and plasma glutamate in autistic children aged 5-16 years matched by age, gender and geography. Regression analysis for the cohort ($n = 99$, 11 females) indicated lower calcium, magnesium, and lithium biomarker levels among affected children; severity of autism was associated with significant regression coefficients for calcium, iron, zinc, and potassium biomarker measurements from red blood cell specimens. All subjects refrained from nutritional supplements for at least two months prior to study enrollment in 2008. However, 29% of cases reported use of psychopharmaceuticals (risperdone and clonidine), and 5-9% reported the use of central nervous system stimulants and/or gastrointestinal medications. Medications like risperdone, clonidine, methylphenidate are known to disrupt metabolic pathways and

therefore affect metabolite profiles and biomarker levels (Adams et al., 2011; Erickson et al., 2005; Evans et al., 2008; Nikolov et al., 2008). Thimerosal, prescribed to control epileptic behavior in autistic children has been shown to affect intracellular calcium levels (Palmieri et al., 2010).

The high use prevalence (40-70%) of psychopharmaceutical central nervous system stimulants and gastrointestinal medications use among American autistic children further complicates blood and urinary biomarker analysis (Adams et al., 2011; Erickson et al., 2005; Evans et al., 2008; Mayer, Padua, & Tillisch 2014; Nikolov et al., 2008; Rosenberg, Mandell, Farmer, Law, Marvin, & Law 2010; Taurines et al., 2010.) Several reviews of nutrient status for autistic children and cases have been conducted with mixed results due to dietary intake, medication and behavioral factors. Underlying food allergies complicated biomarker studies aimed at identifying ASD causality.

Few studies have examined nutrient status and gastrointestinal symptoms in autistic childhood populations not using medications for psychosis or mood disorders. Evans et al. (2008) quantified the urinary metabolic output of autistic children, compared to that of matched (mostly sibling-matched) control children (age 5-15 years; median age 7-9 years) enrolled in two clinics in Australia. Thirty-four autistic children were identified, including 12 untreated cases, and 22 cases receiving various therapies including antifungal treatment for gastrointestinal *Candida* infection, probiotics, injection of secretin hormone to control duodenum pH, and casein and gluten-free diets administered and recorded by parental compliance. Control females had significantly higher levels of urinary glucose and aspartic acid; aspartic acid was three times

significantly higher ($p = 0.05$) than the male control group. No explanation was provided for this finding. The unmedicated autistic group had the lowest level of amino acids excreted, including eight essential amino acids. There were no differences in urinary glucose, sucrose, arabinose or tartaric acid among untreated ASD, treated ASD or the control group. The researchers speculated a possible role of lower phenylalanine and tyrosine with regard to dopamine levels, lower tryptophan levels affecting serotonin levels, and ornithine levels affecting ammonia toxicity or retinal degenerative photosensitivity in untreated autistic patients. The effect of gender on dietary metabolites was reported for glucose and aspartic acid, but these findings were not interpreted.

Other biochemical gender-differences among ASD cases have been reported. Longer light reflect response, smaller and slower pupil constriction has been associated with autism diagnosis and was more symptomatic of boys rather than girls and was proposed to be a diagnostic screening criteria for ASD (Fan, Miles, Takahashi, & Yao, 2009). Gender differences in xenobiotic gene (CYP and GST) expression have been identified in ocular tissue of rats aged 3 to 8 week old (Nakamura, Fujiki, & Tamura, 2005). Female rats tended to have higher levels of CYP gene expression. Within the literature review, only two controlled studies using either children or adults not subjected to psychosis-related medication reported gender differences in metabolic biomarkers (Evans et al., 2008; Mittelstrass et al., 2011).

Therefore, there is limited and inconclusive evidence of the identification of amino acid, urea cycle and serotonin biomarkers associated with autism. There is very limited evidence of gender-associated differences in glucose metabolic markers among

post-natal females with or without autism diagnosis. In addition, based on rodent models, possible early maturity and/or increased PON1 and/or CYP gene function in females may affect the body's ability to clear or detoxify prenatal or infant xenobiotic exposures. Evidence for dietary metabolites associated with autism is complicated by the dual impact of dietary restriction and intervention therapies, as well as food refusal and selective, repetitive dietary choice characteristic of children with autism (Ahearn, Castine, Nault, & Green, 2001; Paterson & Peck, 2011; Ritvo & Freeman, 1978). These factors, and the biostability of many blood or urinary biomarkers confound their use to inform plausible mechanisms of ASD or explain the gender differential of ASD. Therefore, information about metabolites and biomarker measurements for a well-characterized and early life stage restricted diet of breast milk and/or infant formula may inform autism etiology.

Lactation as a Neonatal Biomarker for Autism

The rationale for searching literature for the relationship between prenatal, neonatal and postnatal dietary metabolites was to inform the identification of biomarker candidates for well-characterized metabolic dysfunctions associated with ASD. Prenatal critical timing of environmental exposure and dietary factors associated with ASD were briefly reviewed in the previous section describing critical windows of exposure. The literature evidence for the relationship between childhood blood, urinary, and dietary metabolite factors and ASD was limited and inconclusive as detailed in the previous two sections detailing biomarker candidates and dietary metabolites associated with ASD. It was of interest to review biomarker literature related to a more restricted or controlled

neonatal diet- using lactation as a proxy variable for infant metabolic status. Gender effects on lactation practice were also of interest. A recent symposium on the impact of breast milk and infant formula diet on the nutritional, microbial, and immune status of offspring discussed emerging research topics (James, 2012). But the discussion focused on broad themes and did not address autism or ASD gender risk. The symposium addressed various types and complimentary breast milk and lactation practices.

The World Health Organization, WHO, criteria of exclusive breastfeeding is defined as the following:

no other food or drink, not even water, except breast milk (including milk expressed or from a wet nurse) for 6 months of life, but allows the infant to receive ORS, drops and syrups (vitamins, minerals and medicines). (WHO, 2001)

Adequate maternal health fosters healthy lactation capacity and competence; but nutrient profiles of maternal diet may not be directly transmitted and reflected in breast milk composition (Godfrey & Meyers, 2009; Meldrum et al., 2012). Rather, breast milk reflects a dynamic nutrient composition which differs over time, with maternal hormone status and with infant delivery status (Davis, Nguyen, Garcia-Bravo, Fiorotto, Jackson, & Reeds, 2007; Fidler & Koletzko, 2000; Godfrey & Meyers, 2009; Kuipers, Luxwolda, Dijk-Brouwer, & Muskiet, 2011; Meldrum et al., 2012). Breast milk composition differs from infant formula in many respects, including the inclusion of defensin proteins, IgG, IgM, lysozymes, lower protein content and different polysaccharides, fatty acid, and lipid profiles in human breast milk (James 2012). Walther et al. (2011) reported a high protein weaning diet lead to SIDS-like syndrome in a mice study and suggested fetal

programming mechanisms may adversely affect offspring metabolism. The researchers suggested implications for high protein human maternal diets.

Healthy People 2020 prevalence targets include increased WHO-exclusive breastfeeding (0-3 mo.) to 46%, and 25.5% (0-6 mo.) since breastfeeding affords higher immunity defense against child ear and respiratory infections, dermatitis, gastroenteritis, type 2 diabetes, obesity, SIDS, and fostered skin-to-skin and maternal-child bonding (DHHS, 2007, 2014). Li, Dee, Li, Hoffman, and Grummer-Strawn (2014) reported that infants ($n=1281$) exclusive breastfed by WHO definition beyond 6 months had lower odds of sinus, ear, nose, and throat infections at age six; but breastfeeding practice had no effect on upper respiratory or lung infections. Evidence of lactation benefits to maternal and child health have been reported in several meta-analyses; as have the risks associated with nonbreastfeeding (American Academy of Pediatrics, 2012; Ip et al., 2007; Chung, Raman, Trikalinos, Lau & Ip, 2008; Godfrey & Meyers, 2009; Hauck, Thompson, Tanabe, Moon, & Vennemann, 2011). One meta analysis suggested breastfeeding for any duration may be protective against SIDS risk but the summary odds ratio was estimated to only be $OR= 0.55$, 95% CI [0.44-0.69] for 288 studies conducted from 1966-2009 (Hauck et al., 2011). The specific effects of lactation practice on subsequent offspring autism risk deserves more research attention.

With regard to the relationship of lactation and ASD and the gender differential of autism, few papers were identified in the literature. One paper addressed psychosocial bonding and trust benefits or biomarkers, such as oxytocin signaling in rat models (Higashida et al., 2010). Other papers addressed environmental exposures and risks

related to bottle feeding or environmental pollutants using ecological study designs (Hertz-Picciotto et al., 2011; VanDen Hazel et al., 2006). Chemically derived contaminants such as bis-phenols (BPA) and other plastic additives in processed food can and lid liners, plastic bottles, and baby bottle nipples were listed as potential health risk factors (Olshan, 2007; WHO, 2010).

One case study paper discussed food refusal, breastfeeding and failure to thrive among six infants in Ohio (O'Connor & Szekely, 2001). The effect of gender, and ASD risk was not addressed. Lucas (2011) conducted a retrospective study using a convenience sample of 20 mothers in Illinois to discuss the maternal breastfeeding experiences and behavior of children later diagnosed with ASD. The conceptual framework for the study included behavioral and biological factors with adjustment of socioeconomic status, intrapartum history, and professional breastfeeding assistance. Mothers were categorized based on type of professional breastfeeding assistance during the first month after delivery for 23 full term singleton neonates with birth weights above 2500 grams, and ASD diagnosis before age 11. Mothers completed a survey of socio-environmental questions, a semi-structured interview and postinterview summary. Consistent thematic responses included "insatiable feeding", a vigorous suck that did not stop with satiation, diminished social interaction with mother during lactation, and > 70th percentile for weight gain during the first year. Some researchers have suggested infants with bronchopulmonary dysplasia, cytomegalovirus infections, Prader-Willis syndrome, and ASD have difficulty performing typical suck:swallow:breathe ratios and exhibit periods of apnea, inability to feed, and difficulty with initiation of nutritive suck (Gewolb

& Vice, 2006; Lucas 2011; Miller et al., 2011). However, Lucas (2011) reported adequate first year weight gain among infants later diagnosed with autism.

Field (2014) reported breastfeeding was a risk for ASD among first born males to mothers diagnosed with psychopathology among a case-control study of 112 cases and 139 age and sex matched controls. In the absence of parental psychopathology recorded on medical records, breastfeeding more than twice per day, for at least four months duration was associated with lower risk of ADHD and ASD. Breastfeeding practice did not differ by maternal age, but was less likely to occur after the first-born child, regardless of ASD status. Field (2014) reported 24-29% of the sample was either bottle-fed, or breastfed for less than 4 months.

Three additional research articles quantified lactation proxy variables associated with autism. The articles used different study designs to address metabolic markers, lactation duration and subsequent ASD risk (Ostergard et al., 2011; Schultz et al., 2006; Shamberger, 2011). Some researchers described speculative commentary regarding the pivotal role of fatty acids in human breast milk and reduced autism risk (Brown & Austin, 2009).

Shamberger (2011) conducted an ecological study of the relationship of participation rate in the U.S. Department of Agriculture's Food and Nutrition Service (FNS) for Women, Infant and Children (WIC) to ASD prevalence rates in states and U.S. counties. WIC is a federal grant program for supplemental foods, health care referrals, and nutrition education for low-income pregnant, breastfeeding, and nonbreastfeeding postpartum women, and to infants and children up to age five (FNS, 2012). States with

highest WIC participation had significantly lower ASD prevalence rates ($p < 0.02$). For 21 counties with New Jersey and 30 counties in Oregon, a similar observation was reported ($p < 0.02$ and 0.05 , respectively). Exclusive breastfeeding prevalence had increased from the period 2000 to 2004 but infants who were solely breast-fed tended to have diets with lower thiamine, riboflavin and vitamin D than U.S. minimum daily requirements for these nutrients. Effect of gender on WIC use or ASD was not reported.

An original research article described the effect of breastfeeding duration on ASD in a retrospective, internet based survey conducted between February and April 2005 using 861 children with pervasive or regressive autism and 123 matched controls in the New Jersey-based nonprofit organization, Autism Internet Research Survey (Schultz et al., 2006). Breastfeeding was recorded using nine categories of duration. The nine duration categories were recoded to five categories; less than 2 months, 2-6 months, more than six months, unknown, or none. The researchers reported odds ratio for subsequent autism diagnosis for the absence of breastfeeding, compared to breastfeeding for more than six months was 2.48, 95% CI [1.42, 4.35] for all cases in the case-control design. After limiting the case definition to include only regressive autism, the odds ratio was decreased to 1.95, 95% CI [1.01, 3.78]. Data for parental recall of specific infant formula brand was also collected to discern the use of infant formula with or without docosahexanoic acid and arachidonic (DHA) supplementation. Results suggested the odds ratio of subsequent autism diagnosis for children fed infant formula without DHA fortification versus exclusive breastfeeding was 4.41, 95% CI [1.24, 15.7]. No gender stratified results were reported. Brown and Austin (2009) noted that Schultz et al. (2006)

did not account for colostrum intake, which is known to contain higher levels of immunoglobulins, protein, and polyunsaturated fatty acids (PUFA). The two studies above (Schultz et al., 2006; Shamberger, 2011) did not adjust for maternal nutrition status. Brown and Austin (2009) hypothesized fatty acid deficiencies may be linked to increased autism risk but did not discuss a gender differential.

Ostergard et al. (2011) reported breastfeeding duration, adjusted for infant mean energy intake and diet supplementation was a strong predictor of plasma vitamin D status in a cross-sectional study of nine-month old Danish infants. Eighty-nine percent of infants had concentrations above 20 nmol/L; a minimum threshold level associated with bone health (Humble, Gustafsson, & Bejerot 2010). Plasma vitamin D levels were much higher in these infants than in Danish teen-agers and were much higher than levels reported in a US case-control design of to study the relationship of plasma vitamin D and autism for boys aged 4 -8 years of age (Molloy et al., 2010). The role of vitamin D in autism was recently summarized by Kocovska, Fernell, Billstedt, Minnis, and Gillberg (2012). While the review did not include a discussion of the role of lactation or gender in the association of autism and vitamin D status of mothers and offspring, the researchers discussed the limitations of the Molly et al. (2010) study with regard to the health status of controls. Kocovska et al. (2012) proposed vitamin D status affects brain development and gene regulation. The gender-differentiating role of vitamin D and will be discussed in detail later in this chapter as it has been reported in healthy persons.

There were surprisingly few publications identified which studied the relationship of lactation duration with subsequent ASD. The effect of gender on lactation and

subsequent autism was not discussed in the literature but deserves more attention.

Lactation status may serve as a reliable, valid proxy variable which may inform the relationship of epigenetic (infant diet) factors and subsequent autism diagnosis. Because lactation occurs typically before the age of autism diagnosis, there is a lessened likelihood of metabolic confounding or interaction with pharmacotherapy, food refusal and other dietary restrictions. Lactation status is a noninvasive, readily-recalled factor which aligns well with case-control study designs. Neonatal diet as measured by lactation duration was identified as a result of the literature review as a possible, reproducible, non-biased early proxy variable to study ASD etiology and the gender ASD risk.

Gender-Differentiated Metabolites as Biomarkers

Generalized literature results corroborate the basic metabolic pathways of mitochondrial dysfunction, increased oxidative stress with co morbid neurologic, immunologic, gastrointestinal, and toxicological consequences associated with autism (Abrahams & Geschwind, 2008; Gabory et al., 2009; Hu et al., 2009; James et al., 2010; Ratajczak, 2011; Taurines et al., 2010). Those studies do not account for the critical windows of exposures related to autism onset, or interpret the relative contribution of epigenetic risk factors. Therefore, the literature review was expanded to include publications on genetic, hormonal and behavioral factors affecting metabolic gender-differential of healthy, unaffected children due to the lack of studies specifically focused the relationship of biomarker candidates and male predominance for risk of clinical initial autism diagnosis conducted at age eight years. The plausibility of gender-associated biochemical markers as a main effect variable, rather than a confounding variable in the

relationship to ASD was explored in the literature. Gender-specific metabolic biomarker research among healthy males and female infants and children was identified in the literature as an emerging area of metabolomic study. Gender-associated differences in lipid, carbohydrate and vitamin D metabolism were reported as described below.

Nikkila et al. (2008) reported metabolic lipid profile changes over an 11 year period among healthy children between birth and four years of age stratified by gender. The Finnish cohort study included more than 8,000 children with characterized serum lipid profiles using hidden Markov models. The results indicated the major developmental stage difference between girls and boys was attributed to serum sphingolipids levels at 1 year of age. Girls tended to have higher sphingolipid levels. Sphingolipids are key and common membrane components, resemble phosphatidylethanolamine and phosphatidylcholine with similar electric charge and are present in most membranes and myelin sheath surrounding central nerve cells (Lehninger, 1982). Gender-based progressive trajectories in longer chain triacylglycerols (storage lipids) characterized metabolomics for aged cohorts of 1 to 4 years. Using a healthy Hutterite founder population in a genome-wide association study, Weiss et al. (2006) reported high density lipoprotein-c and triglycerides levels were strongly sexually dimorphic, as was systolic and diastolic blood pressure, body mass index, height, and serotonin levels among 806 adult subjects. It was presumed that these metabolic differences were gender-specific since genetic inheritance changes were controlled and fairly homogenous.

Hagenau et. al. (2009) conducted a global ecological meta-regression analysis of 394 cross-sectional studies on serum 25 hydroxyvitamin D and concluded women had

slightly higher levels than men and Caucasians had higher levels than nonCaucasians. Bolland et al. (2007) reported women had higher vitamin D binding protein levels than men in a cross-sectional cohort but levels were not related to age or adiposity. It was speculated that women had higher serum vitamin D levels because vitamin D binding protein levels rises in response to system estrogen levels. Other researchers also speculated estrogen has a protective effect against low serum vitamin D active metabolite levels in women (Cannell, 2008; Hagenau et al., 2009). Other researchers have hypothesized higher estrogen in females may mediate the toxic effects of excessive glutamate associated with ASD based on theorized mechanisms in rodent models (Pastural et al., 2009). Skin color, sunlight exposure also affect vitamin D levels as reported in studies involving immigration to Northern latitudes (Kocovska et al., 2012).

Mittelstrass et al. (2011) showed significant gender-based differences in lipids, selective amino acids, and six-carbon sugar metabolites. Quantified gender differences were reported for 102 of 131 tested metabolites in a healthy German adult population cohort. Serum levels and gene function associated with sphingolipids, glycine, serine levels were higher; and those associated with hexose sugars were lower in women. This finding was congruent with the research of Evans et al. (2008) who reported teen-aged Australian girls used as controls in the study had higher urinary glucose levels than control boys in a case-control ASD study (see Table 2). But Evans et al. reported no gender-stratified urinary glucose trend for the 12 children diagnosed with ASD not subjected to prescribed medication or restricted dietary intake.

Table 2 summarized biomarker literature identified which reported gender-stratified results for infants and children. Biomarker studies that reported gender-based biomarkers among healthy controls, including healthy adult controls were included in Table 2. The literature search for studies of gender-differential risk in autism resulted in a refinement and narrowed scope such that very few studies, including meta-analyses, reported gender-stratified data or discussion of gender as a covariate within publications addressing autism outcomes.

The data in Table 2 showed seven of 11 publications indicated gender differences in fatty acid and/or lipid related biomarkers, or adipose tissue associated hormones such as leptin. Three studies reported higher levels of serum vitamin D in healthy infants and white women.

Ostergard et al. (2011) reported differences ($p = 0.37$) in plasma active vitamin D levels among infant girls (25-151 nmol/L) and boys (12-150 nmol/L) in a healthy cohort of 255 nine-month old children. Of the 11 studies with gender-stratified results for metabolic biomarker differences, only three studies (Ashwood et al., 2008; Evans et al., 2008; Wiest et al., 2009; Wiest, 2007) involved ASD as the dependent variable. In these three studies of children aged 2-15 years, dietary intake was not standardized or controlled. Biomarkers associated with significant gender and/or ASD were not replicated, and included plasma leptin level, urinary glucose levels, and serum triglycerides. Wiest et al. (2009) reported reduced fatty acid oxidation levels in both autism case and control girls used in the study of 169 children. The authors theorized females metabolize polyunsaturated fatty acids more efficiently than males, regardless of

ASD status; but did not report dietary regime or adjustments made to metabolic biomarker data to account for nonstandardized dietary intake and nutritional status.

Wiest (2007) reported fish intake was monitored, but did not account for fish oil dietary supplementation, which the author suggested could account for mixed results.

Mills et al. (2007) suggested circulating blood DHEA levels may be a biomarker of growth-hormone related metabolism. In a study of 71 boys with ASD screened for steroid-related pharmacotherapy use, blood levels of DHEA and DHEA sulfate did not differ among ASD cases and controls whereas growth-related hormone factors (IGF-1, IGF-2 IGFBP-3 and GHBP) were higher among ASD cases. The effect of dietary supplementation was not addressed in the study. Gender effect was not reported or studied due to the small number of female cases in the sample (Mills et al. 2007).

Table 2

Gender-Stratified Studies of Biomarker Analysis in Infant and Child Cohorts

Researcher	Area and time period	Population enrollment	Percent or number by gender	Autism or outcome criteria	Independent variables	Study design and method	Results
Ashwood et al., 2008	California 2003-2006. MIND, CCEH and CHARGE registries. 169 children.	Age range 2-15 years (mean 4 yrs) 70 autistic (included 37 regressed) 99 aged-matched controls (included 26 siblings and 23 pervasive developmental disorder not otherwise specified (PPDOS))	76-87% boys in controls (78 total; 18 girls) TDs, 20 sibs, 20 developmentally disabled) 86-88% boys in cases. (60 total; 16 girls including 31 with early onset ASD. And 29 regressive)	ADIR(DSM-IV/ICD-9) and an ADOS cutoff value on modules 1 or 2. Four group comparison (ASD, typically developing, or TD, (total disability) and develop-mentally delayed (DD) siblings	Plasma leptin samples, Body mass index measured by mean, and median values per group. Body mass index (BMI) for age Z-scores.	Four group comparison. Rank sums of leptin and log-transformed leptin level. No repeated measures or discussion of effect of pharmacotherapy	Leptin levels were significantly higher in early onset ASD compared to controls, siblings and children with delays (TD, DD). Leptin level was not associated with BMI or BMI for age Z-score
Bolland et al., 2007	New Zealand	Age 38-85 Healthy adults	50 men 50 women	Healthy controls	Plasma vitaminD (25-OH-vitaminD)	Group comparison	Women had higher vitamin D & Vitamin D binding protein-not related to BMI or age
Evans et al., 2008	Australian medical clinic	Age range 5-15 years (mean 7-9) Excl. Asperger, ADD, ADHD 34 cases 29 control	45% boys in controls (29 total; 16 girls) 91% boys in cases (34 total; 3 girls) 22 of 34 cases on medicine or diets	DSM-IV assessed by 2 physicians	First urine morning midstream urine. Urinary amino acids, tartaric acid, and sugars analyzed	Group comparison. Amino acids and relative abundance (to correct for urine protein level)	Control females had significantly higher aspartic acid and glucose in urine.

Researcher	Area and time period	Population enrollment	Percent or number by gender	Autism or outcome criteria	Independent variables	Study design and method	Results
Hagenau et al., 2009	Meta analysis 1970-2004	394 published global studies	Age 0-75 years 277 reports for women; 105 reports for men	Healthy Controls	Serum 25-OH-vitamin D	Meta-analysis and Meta regression	Women had higher mean serum Vita D levels, followed by Caucasian men
Kuipers et al., 2012	African fetuses	Fetuses postmortem	Not gender stratified	Gestational Age	Fatty acids		3rd trimester differences
Mittelstrass et al., 2011	German Cooperative Health Research in the Region of Augsburg, KORA cohort	Cardiovascular population Adult registry	1452 males 1552 females	Not Applicable	131 Serum biomarkers; amino acids, phosphatidyl-choline, sphingo-myelin, acyl-carnitine, C6-sugar	Group comparison Linear regression. Bonferroni-estimate on gene single nucleotide polymorphism (SNPs)	Females had higher serine, glycine, lower C6-sugar, higher phingolipids. GWAS (p< 0.05) for carbamoyl-phosphate synthase1 region
Ostergard et al., 2011	Denmark SKOT cohort	Infant (9 mo) civil registration	128 males 127 females	Plasma Vitamin D	Lactation, BMI, Supplements	T-tests & regression	Girls had higher Vita D
Pastural et al., 2009	Minnesota clinic (Jonty Foundation) 1 yr study. 4/15 cases on carnitine	Age range 2-10 yr 12 controls 15 cases	100% male cases (15 total) 75% male controls (12 total; 3 girls)	DSM-IV	Fasting plasma taken each 6 mo. polyunsaturated fatty acids, very long chain fatty acid, amino acids, glutamate	Group Comparison	Cases had higher levels of poly-unsaturated fatty acids and ethanolamine phospholipids. Control girls: higher glutathione, cysteine, and homocysteine.

Researcher	Area and time period	Population enrollment	Percent or number by gender	Autism or outcome criteria	Independent variables	Study design and method	Results
Nikkila et al., 2008	Finland Type 1 Diabetes Registry Longitudinal (1994-2006) 8000 children	Age 0-4 years	27 boys 32 girls	Healthy Controls	Serum lipid profiles tested at 3 month intervals to confirm no progression to Type 1 diabetes (11 samples/child)	Bayesian based hidden Markov model to biotransition stages	Girls had higher sphingolipids at age 1. Medium chain triacylglycerols at age 1-2 yrs and higher long chain triacylglycerols age age/stage 2-4 years.
Novak & Innis, 2012	Canada Healthy subjects	631 (233 pregnant) and 313 children	27 % males Incl. 150 boys	EPA, DHA intake By dietary recall	Pregnancy, gender, age	ANOVA	EPA &DHA intake associated with protein intake
Weiss et al., 2006	US Hutterite 806 people Mean age 29 (range 6-89)	Inbreed coeff; approximately 1.5 cousins)	567 complete profiles collected with 95% response	Not Applicable	17 GWAS tests for morning serum collected over two winter periods	Monte Carlo simulation	Lipids, blood pressure, FEV, Eos, IgE, lymph cortisol, serotonin differed by sex
Wiest et al., 2009; Wiest, 2007		See Ashwood et al 2008 above	See Ashwood et al., 2008 above	See Ashwood et al., 2008 above	30 Plasma fatty acids in 7 classes. One blood draw. Raw and log-transformed data.	Group comparison Linear mixed effect model	ASD+females had lower 20:4n-6 triglycerides than control females. All girls: < fatty acid oxidation

Note. ADD= attention deficit disorder; ADHD= attention deficit hyperactivity disorder; GWAS= genome wide association studies; EPA= eicosapenaenoic acid; DHA= docosahenaenoic acid; FEV= forced expiratory volume; Eos= eosinophils; IgE= immunoglobulin E.

Childhood dietary nutritional factors have been shown to affect biomarker studies in autism research, as summarized previously in this chapter. A comparison of two case-control childhood studies and two cross-sectional adult studies (Fernell et al., 2010; Humble, Gustafsson, & Bejerot, 2010) which measured the effect of plasma vitamin D on autism diagnosis among children and adults with autism showed mixed results (Kocovska et al., 2012). Health status of controls, maternal plasma vitamin D levels and severity vitamin D deficiency interacted and complicated results; and none of the four studies indicated breastfeeding history or duration as a study variable or confounder in the dose-response relationship to ASD (Kocovska et al., 2012). Case-control studies for the childhood cohorts in China, Vietnam, Egypt, and the U.S. have been discussed in this literature review (Gong et al., 2014; Hanieh et al., 2014; Meguid et al., 2010; Molloy et al., 2010). Neither the four childhood studies, nor two adult studies on the effect of vitamin D status on autism reported the effect of gender on health status or the effect of gender on ASD. Vitamin D status may not be a reliable, biostable, standardized proxy biomarker for ASD or ASD gender-differential risk.

Neonatal diet data measured by the proxy variable of breastfeeding status, may provide a more consistent, reproducible relationship with autism status and inform the gender differential in autism diagnosis. Ostergard et al. (2011) reported breastfeeding duration, adjusted for infant mean energy intake and diet supplementation was a strong predictor of plasma vitamin D status. For these reasons lactation duration may be an adequate, reproducible surrogate marker for neonatal nutritional and health status. Ronald, Happe, Dworzynski, Bolton, and Plomin (2010) reported lack of breastfeeding

was a consistent risk factor for neonatal complications in twins. Breastfeeding intention, capacity, and competence influence lactation duration (DiGirolamo, Thompson, Martorelli, Fein, & Grummer-Strawn, 2005; Lucas 2011; McCann, Baydar & Williams, 2007). Breast milk composition differs over time with maternal hormone and infant delivery status (Davis, Nguyen, Garcia-Bravo, Fiorotto, Jackson, & Reeds, 2007; Fidler & Koletzko, 2000; Godfrey & Meyers, 2009; Kuipers et al., 2011; Meldrum et al., 2012). Nutritional status of lactating mothers influences infant health status (Mariani, Chabies, Jeziorski, Ludwig, Lalande, & Rodiere 2009; Shamberger, 2011).

It is plausible that prenatal maternal nutrition affects epigenetic pathways, placental transfer, and gender-specific risks associated with autism spectrum disorder. Rogers (2008) speculated that enhanced maternal folate status may increase survival rates of particular genotypes with subsequently higher postnatal requirements for methylation needed for typical neurodevelopmental growth. Schmidt et al. (2012) reported low parental folic acid intake was associated with ASD risk, mediated by particular maternal methylene tetrahydrofolate reductase (MTHFR) gene alleles. Maternal lipid, protein, vitamin D, and other nutrient intakes may affect fetal development, as well as breast milk composition. It is of interest whether there are gender-specific effects of these metabolites which may inform the ASD gender differential. The effect of gender on perinatal diet, lipid, vitamin D and ASD is largely understudied.

The speculative role of estrogen in gender-differentiated metabolism has far-reaching consequences (Bolland et al., 2007; Cannell 2008; Hagenau et al., 2009; Hu et al., 2009). Hu et al. studied nonaffected sibling and AGRE ASD cases and concluded

gender differential risks may be associated with cholesterol/steroid metabolism at the androgenic hormone level, based on differences in genetic expression profiles.

Interactions of biochemically based maternal and paternal factors may have heretofore unrecognized effects on the gender differential pattern associated with autism.

Observable, consistent, and readily documented or recalled gender-based factors may provide robust analysis of the effect of these prenatal factors on the gender differential of autism. Retrospective, externally validated prenatal risk factors, including parental age, health status, parental risk behaviors, pregnancy and delivery complications, and familial genetics are likely also associated with epigenetics of autism. These factors may also help to explain the gender-differential risk associated with autism onset. Suggestive, recurrent literature evidence for prenatal risk factors which indicate gender specific effects on autism diagnosis are addressed in the next section.

Prenatal, Gender-Associated Risk Factors for Autism

Evidence supporting a plausible gender-differential risk factor profile was related to variables of parental age, SIDS type symptoms, and infant hypoxia risk, parental smoking status, obstetric or pregnancy complications, perinatal maternal diet as it affected birth outcome and offspring epigenetics, and parental genomic analysis.

Independent variables amenable to retrospective, direct observation, direct measurements or externally validated recall measurement were the primary focus of the literature review on this topic. These study topics are summarized below. The ASD risk factors of infant hypoxia, preterm birth, preeclampsia, vitamin D intake, estrogen levels, xenobiotic clearance liver enzymes, and lactation are described.

Parental Age

Relationship of pregnancy outcomes, and developmental risks including autism have been generally identified in the literature to be “U”shaped with regard to parental age for U.S. and European populations. At young age (i.e. less than 20 to 25 years) increased risks of suboptimal parental health and behavioral risk profiles appear to influence pregnancy status (Anello et al., 2009; Cantor et al., 2007). At increasing age (i.e. greater than 35-50 years), cumulative behavioral, nutritional and/or environmental exposures affected fertility as well as pregnancy outcomes (Anello et al., 2009; Durkin et al., 2008; Field, 2014; Puleo, Reichenberg, Smith, Kryzak, & Silverman 2008; Lundstrom et al., 2010). Jenkins (2013) reported age-associated alterations to sperm DNA methylation at the global and cytosine phosphate guanine (CpG) levels with increasing methylation in DNA regions biased toward hypomethylation. This finding was in stark contrast to somatic cell age-associated methylations.

Lauritsen et al. (2005) estimated higher relative risk (*RR* 1.7) of autistic offspring for maternal age for mothers 12-19 years old, lowest risk for maternal age 25-29 year (*RR* 1.1) and increased relative risk (*RR* 1.2) of autistic offspring for mothers aged more than 30 years. Lundstrom et al. (2010) reported a U-shaped risk associations for paternal age and autism diagnosis in U.K. and Swedish cohorts recruited from 1992 to 1998. Each cohort contained more than 11,000 subjects, with childhood autism diagnosis at age 9-12 years. Paternal age group categories were: less than 25, 25-34, 35-44, 45-50, and greater than 50 years. Paternal age less than 25 years or greater than 50 years was associated with unadjusted odds ratio of 2.5 and 3.2, respectively. After adjustment for maternal age,

zygosity and socioeconomic status, adjusted odds ratio were 1.9 and 3.4, respectively for father age-cohorts younger than 25 or older than 50 years. Reichenberg et al, (2006) reported autism risk increased from 10 to 107 per 10,000 births over five increasing paternal age groups in an Israeli birth cohort ($N = 318,506$) over a six year study period in the 1980's. A ten year increase in maternal age was associated with a 38% increase in odds ratio for subsequent autism diagnosis in offspring in a California cohort of singleton births born from 1989 – 2002 to mothers from age 15 to 44 years; median age 27-28 years (Grether, Anderson, Croen, Smith & Windham 2009). In the same study, a ten year increase in paternal age was associated with a 22% increase in odds ratio for fathers from age 15-64 years (median age 29 -31 years). Similar adjusted odds ratio (1.3) was found for 10 year increases in maternal and paternal age in a smaller California cohort recruited from 1995 to 1999 (Croen et al., 2007). Offspring sex-differences were not significantly different in the 2007 study, but there was a higher relative risk of autism diagnosis in girls, $RR = 1.55$, 95% CI [0.93-2.59] than boys, $RR = 1.27$, 95 % CI [1.01-1.60] as a function of increasing maternal and paternal age. Field (2014) reported higher risk of ASD among female offspring with increased parental age but the trend was not found for male offspring ($n=112$ cases).

Durkin et al. (2008) reported that after adjustment for the other parent's age, birth order, maternal education and other covariates, both paternal and maternal age were independently associated with offspring autism at age eight for the Center for Disease Control and Prevention's Autism and Developmental Disabilities Monitoring Network of ten geographic areas for all 326,785 livebirths in 1994. Adjusted odds ratio for maternal

age of 35 years or greater was 1.3, 95% CI [1.1, 1.6] and adjusted odds ratio for paternal age of 40 years or greater was 1.4, 95% CI [1.1, 1.8]. Frequency of gestational age less than 37 weeks averaged 12.5 -13.6% for ASD cases and 8.7-9.8% for the full cohort. Among 2,142 cases defined by ADOS criteria, 81.2% were male offspring and 32.4% of children had intellectual impairment (I.Q. less than 70). Firstborn infants of two older parents were three times more likely to develop ASD compared to third or later born offspring of moms aged 20-34 years and fathers less than 40 years old.

Anello et al. (2009) reported the typical male:female ratio of autism diagnosis (4:1) reflected a U shaped distribution as a function of paternal age in an AGRE cohort. The male:female ratio ranged from 6.2 for fathers less than 30 years old, to 3.3 for fathers aged 40-44 years in a U.S. cohort of 393 cases in families with two or more diagnosed autism cases using a “strict”, ADI-R based case definition. Trends were not affected by adjustment for maternal age.

Therefore, the literature suggests paternal and maternal age were independent risk factors of ASD among offspring and generally showed less risk of diagnosis for parents aged 25-40 years of age. In two studies, the male-female gender differential risk ratio decreased with increasing parental age (Anello et al., 2009; Croen et al., 2007). Risk of autism diagnosis was found to be higher among first born children compared to multiparous families for parents in the age range of 20 to 40 years (Durkin et al., 2008). The impact of assisted reproductive health in older adults, and parenting techniques are other parental age-related factors which may affect ASD prevalence (CDC 2012; Schieve et al., 2011). At young age (i.e. less than 20 to 25 years) increased risks of suboptimal

parental health and behavioral risk profiles appear to influence pregnancy status (Anello et al., 2009; Cantor et al., 2007). Younger parents may be more likely to engage in risky behaviors such as smoking and drinking. Anderson, Johnson, and Batal (2005) reported smokers were more likely to be single, non-Hispanic, less educated and were significantly more likely to report alcohol use during pregnancy in a U.S. study. The research also showed that while parenting changes in infant sleep position has decreased the overall rate of sudden infant death, attributed risk associated with maternal smoking and SIDS has increased from 50% to 80% in the study conducted from 1989-1998 (Anderson et al.). Maternal smoking prevalence was not different by infant gender, but odds ratio of SIDS death was two-fold for infant males (Anderson et al., 2005).

Sudden Infant Death and Infant Hypoxia Risk

Prevalence. Stable gender differential prevalence (i.e. male predominance) has been replicated in ASD and for other pervasive developmental disorders (PPD) outcomes. Increased prevalence rates for the more narrowed definition of autism have been implicated worldwide since 2000 (Elsabbagh et al., 2012). It is plausible that underlying pathobiological mechanisms may affect increased prevalence rates and differential gender risk ratios for many developmental delays; including ASD. Differential gender diagnosis risk ratios have been reported to be five- or six-fold higher for other pervasive developmental disorders (PPDs), including dyslexia, Tourette, Asperger, and Timothy syndromes in the U.S. and Europe (Baron-Cohen, Lombardo, Auyeung, Ashwin, Chakrabarti & Knickmeyer, 2011; Bauermeister et al., 2007; Chakrabarti & Fombonne 2005). Perhaps genetic or biochemical gender-associated biological susceptibility or

fetal programming may explain broader PPD and more specific autism risk prevalence. Shared or inherent gender-associated profiles may have common mechanisms, pathways or biomarkers.

The prevalence of sudden infant death syndrome (SIDS) has persistently shown a 3:1 male-to-female mortality risk among infants despite behavioral interventions to promote supine sleep position for infants of both sexes in the United Kingdom, Scandinavia, U.S. and Australia (Mage & Donner, 2007; Mitchell & Stewart, 1997). There are similar or overlapping risk factor and biochemical dysregulation mechanisms documented for SIDS and ASD (Habek & Kovacevic, 2011; Kinney & Thatch, 2009). The critical development period of SIDS risk appeared to be age two-four months when brain weight typically doubles (Carolan & Bye, 2011). Low birth weight, smoke exposure or parental smoking, apnea, previous episodes of interrupted breathing, and hypoxia are SIDS risk factors (American Academy of Pediatrics, 2011; Goldwater, 2011; Van Norstrand & Ackerman, 2010). SIDS-related risk factors appear to more adversely affect male infants than female infants independent of race/ethnicity (Kinney & Thatch, 2009). Gender-stratified analysis of prenatal, neonatal and infant exposures may inform evidence for overlapping or common risk profiles for subsequent SIDS and ASD.

Mechanisms. Burstyn et al. (2011) studied the association of fetal hypoxia at birth and subsequent autism diagnosis by ICD-9 criteria and medical assessment among a population based cohort born between 1998 and 2004 in Canada. The researchers reported an excess ASD risk among males (*OR* 1.0 to 1.6) who were hypoxic at birth for premature and full-term infants diagnosed with fetal hypoxia. Plausible explanatory

hypotheses for hypoxia-ASD relationship described in the study publication included prenatal dopamine (serotonin inhibitor) exposure, maladaptive stress responses and/or gender-specific placental physiology (Burstyn et al., 2011).

The interrelationships of smoke exposure, brainstem development, serotonin, and dopamine regulation are an active area of SIDS research (American Academy of Pediatrics, 2011). Croen, Grether, Yoshida, Odouli, and Hendrick (2011) reported maternal serotonin reuptake inhibitor use, mediated by maternal allele form, for up to 12 months, and in particular, the first trimester of pregnancy significantly increased the risk of ASD in offspring which aligned with the theory serotonin inhibition may be associated with hypoxia risk (Burstyn et al., 2011; Habek & Kovacevic, 2011; Previc, 2007). Certain genetic polymorphisms of fatty acid oxidation have also been associated with SIDS (Kinney & Thatch, 2009). However serotonergic (5-HT) genetic alleles responsible for infant gasping response were not associated with SIDS in a U.S. case control study of 96 gender and ethnicity matched pairs (Rand, Berry-Karvis, Fan, Weese-Mayer, 2008). Liu and Deneris (2011) reported transcriptional control of serotonin 5-HT receptor deficiency associated with SIDS occurred before central nervous system neural circuitry formed. Complex multiple weak stressors or underlying congenital factors may impact SIDS.

Like autism, SIDS pathophysiology is presumed to include a convergence of risk factors. The heritable contribution of serotonin transport, cardiac channelopathies, and autonomic nervous system genetic regulation and SIDS outcome is under investigation (American Academy of Pediatrics, 2011). Infant congenital heart defects have been associated with chromosome X-linked disorders and particular genes such as ZIC3,

HTX1 and HTX which may be coincidentally associated with hypoxia and SIDS (Bailliard & Anderson 2009; Gioli-Perira, Pereira, Bergara, Mesquit, Lopes, & Krieger, 2008; Kinney & Thatch, 2009). However, pediatric heart defects have also been associated with epigenetic mechanisms affecting unique delivery outcomes such as low birth weight and prematurity (Zhu, Bonnet, Boussion, Védie, Sidi, & Jeunemaitre, 2007). Therefore, serotonin and dopamine dysregulation may be associated with hypoxia; which may be a risk factor for SIDS and ASD (Previc, 2007). Nijmeijer et al. (2010) reported maternal smoking was associated with ASD and ADHD symptoms but the relationship was mediated by the maternal genetic allele form of COMT and serotonin transporter (SLC6A4) genes. The effect of maternal allele on ASD or ADHD case gender was not reported. In a review of seven large population cohort studies which are detailed below, Kolevzon, Gross and Reichenberg (2007) concluded birth weight, gestational age, and intrapartum hypoxia were significant risk factors for ASD. The common plausible pathobiology of SIDS and ASD has not been fully explored. Maternal smoking during pregnancy is a common risk factor for fetal hypoxia which may be associated with placental insufficiency (Habek & Kovacevic, 2011). The relationship of infant breathing and sleeping disturbance as proxy variables for ASD risk has not been extensively studied, and not studied at all using gender-stratified sample populations.

Parental Smoking Status

Prevalence. Smoking continues to be the predominant behavioral risk concern for pregnant women and women of reproductive age. In the U.S., an estimated 20 to 30% of pregnant women may smoke (Cnattingius, Haglund, & Meirik, 1988; DiFranza & Lew,

1995). In a separate U.S. study, 20% of pregnant women denied smoking, but had high urinary cotinine levels suggesting self-reporting bias and/or environmental smoke exposure (Ford, Tappin, Schluter, & Wild, 1996). In this section, the results of a literature search for *autism, smoking* prevalence, and related biochemical impacts are described for articles which addressed attention-deficit hyperactivity disorder (ADHD) as well as ASD, and SIDS outcomes.

In cross-sectional studies, the association of parental smoking, and in particular, maternal smoking and ASD among offspring has shown mixed results for studies in the U.S., Europe and China (Kalkbrenner et al., 2012; Lee et al., 2012; Mann et al., 2010; Zhang et al., 2010). Measurement or recall bias of smoking behavior was theorized to be strongly confounded by maternal education level, other socioeconomic variables, and mode of data collection (i.e. birth certificates versus medical records) in several studies (Dietz et al., 2011; Kalkbrenner et al., 2012; Lee et al., 2012; Burstyn, Lee, Gidaya, & Yudell, 2012; Vinikoor, Messer, Laraia, & Kaufman, 2010; Zhang, et al., 2010). Nondisclosure of smoking tended to be higher for younger women (age 20-24 years) in a U.S. cohort which quantified blood cotinine concentration to validate smoking status (Dietz et al.). The association of maternal smoking during pregnancy and offspring ASD diagnosis appears to be strongest for higher functioning ASD and for the broader case definition of autism (Kalkbrenner et al.; Lee et al.). Even when large sample sizes were used, cross-sectional studies, case-cohort and case-control designs did not address gender specific risks associated with maternal smoking and autism.

Kalkbrenner et al. (2012) conducted a population-based case-cohort study of 633,989 children, including 3,315 confirmed ASD cases actively registered in the CDC Autism and Developmental Disability Monitoring Network and born in the U.S. between 1992 and 1998. The study objective was to estimate the association between maternal smoking during pregnancy and ASD. Maternal smoking during pregnancy was reported for approximately 13% of the source population and 11% of the population with ASD affected children. The prevalence ratio of maternal smoking was adjusted for maternal education, race/ethnicity, marital status and maternal age. The researchers reported no association between maternal smoking during pregnancy and ASD. When the broader case definition of ASD was used, there was a slightly higher ASD prevalence ratio 1.26, 95% CI [0.91, 1.75], but the trend was not statistically significant. The effect of child gender on prevalence was not reported. Burstyn et al. (2012) cautioned that the researchers did adequately control confounders or assess the impact of smoke exposure misclassification. A conclusion that maternal smoking does not affect fetal development is counterintuitive. A review of the biochemical impact of direct, first-hand maternal smoke exposure suggests evidence for an association with ASD.

Pathobiology. In healthy individuals, acetylcholine, released in the brain which activates neurons in the peripheral nervous system then activates cholinergic receptors to coordinate respiration, maintain heart rate, memory, alertness, and muscle movement (American Academy of Pediatrics, 2011). Nicotine has chemical homology to acetylcholine and therefore with repeated exposure, disrupts brain neurotransmitter receptor number and sensitivity (Rosenthal & Weitzman, 2011; Wang, 2007, p 9-10;

Xuei, Flury-Wetherill, Bierut, Dick, & Nurnberger, 2007; Zhang, Kranzler, Weiss, Xinguang, & Brady, 2009). Disruption may cause minor muscle tremors which releases adrenalin in glands which increase coronary blood flow, heart rate, blood pressure and skin vasoconstriction (Wang 2007) and releases pleasure-associated dopamine in nucleus accumbens. In the case undeveloped or dysfunctional synaptic receptors, or lack of neurotransmitter connections, tics and minor muscle tremors may be a phenotypic biological outcome expression (Meany, 2010; Xuie et al., 2007). There is speculation that maternal smoking affects acetylcholine receptor functions which in turn affect infant brain neuro-development (Duncan, Paterson, & Kinney 2008; Slotkin, 2004; Habek & Kovacevic, 2011; Slotkin, MacKillop, Rudder, Ryde, Tate, & Sedler 2007; Soothill, Morafa, Ayida, & Rodeck 1996). Nicotine reaches the brain within 10 seconds, and has been identified in breast milk and umbilical blood of newborns (Wang-Sattler et al., 2008). Metabolic profiles of non-smokers, former and current smoking adults showed current smokers had lower sphingomyelins and acyl-alkyl-phosphatidylcholines suggesting loss of peroxisomal enzyme activity in smokers (Wang-Sattler, et al.).

Acetylcholine imbalance has been documented in male children with ADHD (Coccini, Crevani, Rossi, Assandri, & Balottin, 2009). The researchers reported reduced monoamine oxidase type B activity which may affect acetylcholine and monoamine balance in unmedicated affected boys but not girls in an Italian sample of 44 children. In general, female resistance to hypoxia has been shown to reduce infant mortality rate by 19-25% relative to males in children aged 1-4 years (Taket, 1986). Fowler, Cassie, Rhind, Brewer, and Collinson (2008) reported liver concentration of polycyclic aromatic

hydrocarbons and blood cotinine were confirmatory biomarkers of maternal smoking status among 69 UK mothers during second trimester of pregnancy. For a smaller paired test using genotyped mothers, among 30 gene candidates representing hedgehog pathway signaling, xenochemical signaling, steroidogenesis (CYP liver-xenobiotic clearance enzymes), endocrine signaling, transcription, Wnt pathway signaling, development and growth, hyposadias and testis descent), only desert hedgehog gene product in Sertoli cells differed significantly between 12 controls and 10 smoking mothers.

Smoking is also associated with increased SIDS rates, despite the uncertain biopathology of SIDS (American Academy of Pediatrics, 2011; Habek & Kovacevic, 2011; Young, Watson, Ellis, & Raven, 2012). SIDS risk increased significantly for mothers who smoked more than 6 cigarettes per day (Shellscheidt et al., 1997 in Habek and Kovacevic, 2011). Anderson, Johnson, and Batal (2005) reported the risk of SIDS was two-fold for mothers who smoked despite promotional campaigns and behavioral reinforcement of placing infants on their backs, without co-bedding during sleep. McDonald et al. (2006) reported maternal smoking elevated umbilical cord levels of adrenocorticotropin hormone among 104 infants delivered by elective caesarean section. The authors speculated cigarette smoking may be associated with hypoxia-related events as a result of hypothalamic-pituitary-adrenal axis involvement in fetal programming responses resulting in increased caroxyhaemoglobin, reduced placental oxygenation, and uterine vessel vasoconstriction. Burstyn et al. (2011) concluded fetal hypoxia was a main effect variable in the relationship to autism after adjustment for socioeconomic status,

birth year, and fetal gender. Hypoxia status and neonatal testing prevalence for hypoxia was skewed to infant males (Burstyn et al., 2011).

Mechanisms. Slotkin et al. (2007) showed permanent, sex-selective cholinergic hypoactivity response (more pronounced in males) in rat pups exposed to second-hand smoke. Nicotine and cotinine have been shown to cross the placenta and accumulate in fetal tissue in both animal and human models (Barnea 1994; Koren 1995; Jauniaux, Gulbis, Acharaya, Thiry, & Rodeck, 1999). Prasodjo et al. (2014) reported that prenatal direct maternal smoking or secondhand smoke at 16 weeks gestation resulted in blood cotinine levels of 0.012 to 0.224 ng/ml and tended to be associated with low whole blood folate levels in women enrolled in an home environmental study in Ohio. Among mothers who smoke after delivery, postnatal exposure of nicotine and cotinine is estimated to be two-to three fold higher in breast milk than in maternal plasma or skin (Onuki et al., 2003; Sastry, Chance, Hemontolor, & Goddijn-Wessel, 1998). Gray et al. (2010) reported that among 87 mothers who self-reported tobacco use in the third trimester of pregnancy, nicotine, cotinine and nicotine metabolites were identified in meconium, the first neonatal feces samples for newborns. Gardnener, Spiegelman, and Buke (2011) reported aspirated meconium and feeding difficulties, fetal distress, umbilical cord complications, hyperbilirubinemia, neonatal anemia and delivery complications were associated with ASD in a meta-analysis of forty case-control studies. These epigenetic and neonatal findings provide suggestive evidence of biological plausibility of specific prenatal gene transcription and allelic expression as a consequence of in utero smoking exposure associated with reduced maternal peroxisome activity, lower airway responsiveness,

hypoxia, and xenobiotic clearance which are plausible risk factors associated with sudden infant death and ASD, but do not specifically address the gender differential of ASD.

Increased levels and activity of genes associated with xenobiotic clearance (i.e. PON1, CYP and GST genes) have been found in female as compared to male rat, hamster, and mice models (bin Ali, et al., 2003; Feingold et al., 1998; Thomas-Moya et al., 2006). Ronald et al. (2010) reported smoking and non-breastfeeding have been consistently identified as risk factors for prenatal and neonatal complications and autistic and Asperger syndrome in U.S. twin populations.

Maternal daily smoking, particularly in early phases of pregnancy (up to 22 weeks gestation) has been associated with increased ASD risk in offspring in Swedish and American cohorts (Hultman, Sparen, & Cnattingius, 2002; Rodier, Ingram, Tisdale, Nelson, & Romano, 1996; Steenweg-de Graaff, Ghassabian, Jaddoe, Tiemeier, & Roza, 2014; Stromland, Nordin, Miller, Akerstrom, & Gillberg, 1994). Mann et al. (2010) reported maternal antenatal tobacco use was associated with increased odds $OR= 1.02$, 95% CI [0.80, 1.30] of ASD in offspring but male gender ($OR 5.68$) and pre-eclampsia, $OR= 1.85$, 95% [1.38, 2.47] were stronger predictors of autism than maternal smoking for multivariate modeling of 13 risk factors among 691 cases and 80,000 controls in a retrospective cohort of births in a South Carolina Medicaid registry. The researchers concluded birth weight mediated the association of preeclampsia and autism.

Animal model research, placental transfer theories, and intrauterine or fetal epigenetic mechanisms may support the finding by Coccini et al. (2009) that xenobiotic clearance of smoke exposure appeared to be slower or less efficient among boys with

diagnosed ADHD. Fetal exposure to smoke has been implicated in gender-differentiated risks of infant hypoxia (Burstyn et al., 2011; Taket, 1986). Lower gene expression of hedgehog regulatory genes in Sertoli cells associated with male testis development was also shown to be affected by smoke exposure (Fowler et al., 2008). There is evidence that glutathione-transferase (GST) gene expression in maternal erythrocytes, lung and placental tissue support xenobiotic clearance of smoke and may cross placental barriers (Watson, Stewart, Smith, Massey, & Bell, 1998). Grazuleiciene, Danileviciute, Nadisauskiene, and Vencloviene (2009) reported mothers who smoked had significantly higher odds ratio, $OR = 3.3$, 95% CI [0.6-18.4] of intra-uterine growth restricted offspring when the mothers also had particular allele forms of two key glutathione-transferase enzymes (null GSTM1 and GSTT1). Wilhelm-Benartzi et al. (2012) studied epigenetic markers associated with maternal smoking and alcohol consumption among 380 pregnant U.S. women. The researchers reported in utero exposure to tobacco smoke and alcohol modified methylation gene markers in placental tissue and suggested epigenetic alternations associated with exposures mediated placental function in support of the fetal programming hypotheses. The effect of in utero exposure to primary or second-hand smoke on fetal development and autism risk deserves more attention. The plausible effect of placental transport of prenatal exposures may provide further insight into ASD etiology. Pedersen et al. (2013) reported maternal smoking, exposure to secondhand smoke during pregnancy, and ethylene oxide exposure was associated with DNA placental cord adducts which may affect intrauterine growth. Maternal reported consumption of fruits and vegetables was associated with lower frequency of DNA

placental cord adducts in a study of 229 mothers and 612 European children. Additional research on proxy variables or biomarkers of obstetric placenta function and placental transport mechanisms on infant traits, ASD and the ASD gender differential is needed.

Obstetric Complications

Types. Increased cesarean section prevalence, placental dysfunction, and other obstetric complications may affect secular and pregnancy-specific outcomes associated with ASD (Gardener, Spiegelman, & Buka, 2011; Guinchat, Thorsen, Laurent, Cans, Bodeau, & Cohen, 2012). Preterm birth, low birth weight, preeclampsia, caesarean section, and delivery complications are common birth outcome risk factors. Preterm delivery is generally predicated by adversely affected maternal vascular health (Tiedje et al., 2008). Low birth weight (< 2500 grams) and preterm birth of less than 33 weeks (Dawson, Glasson, Dixon, & 2009), and caesarean section have been associated risk factors for ASD and higher odds ratio of other PDDs (Gialloreti, Benvenuto, Benassi, & Curatolo, 2014; Langridge et al., 2013; Mann et al., 2010; Schnedel & Bhasin, 2008; Wilhelm-Benartzi et al., 2012). Maternal diabetes, hypertension, and obesity were associated with higher risk of PPD and ASD (Dodds et al., 2011; Krakowiak et al., 2012; Langridge et al., 2013; Neggers, 2014; Tordjman et al., 2014). Cesarean delivery was also associated with ASD risk in cases without intellectual disability in an Australian population cohort study (Langridge et al.). Pregnancy complications have been indicated in autistic offspring (and their nonaffected sibilings) in a study of Australian children (Glasson et al., 2004; Langridge et al.) and in U.S. cohorts (Bilder et al., 2009, Korelinger et al., 2004; Krakowiak et al., 2012). Premature infants were up to four times more likely

to develop autism and have up to an eight-fold autism risk ratio if the mother had preexisting, preconception allergies (Theoharides, Angelidou, Alysandratos, Asai, Fracis, & Kaolgeromitros, 2011). Thirteen obstetric and neonatal factors including abnormal presentation, birth injury or trauma, umbilical cord complications, maternal hemorrhage, low birth weight, infant size, Apgar score, and hyperbilirubinemia were positively associated with ASD in a meta-analysis of 60 risk factors (Gardener et al. 2011).

Guinchat et al. (2012) postulated risk factors for PPDs including autism may be associated with improvements in obstetrical and neonatal management which have increased survival rate of infants with preexisting brain damage. Offspring gender effect on obstetric risk is unclear since complications may be birth-specific.

Diagnosis. Early detection of preeclampsia and jaundice risk is a primary intervention focus in pregnancies in all countries because preeclampsia is a leading cause of maternal and perinatal morbidity and mortality. Preconception health of women likely affects the course of pregnancy and fetal development. Preeclampsia tends to present in late term with a mild clinical course; but maternal complications may include gestational hypertension, renal failure, proteinuria, and edema (Myatt & Webster 2008, Scifres, Catov, & Simhan, 2011), hemolysis, elevated liver enzymes, and low platelet counts (Kuc, Wortelboer, Rijn, Franx, Visser, & Schielen, 2010) with increased risk of later life diseases in mothers and offspring (Aris, Benali, Ouellet, Moutquin, & Leblanc 2009). Pre-pregnancy weight, maternal pulmonary, heart, renal disease and anemia were reported as key non-genetic risk factors of ASD in a Canadian case-control study of 924 cases (Dodds et al., 2011). Gregory et al. (2013) reported maternal diabetes was a

significant preconception risk factor affecting ASD in a U.S. case-control population cohort. It was of interest whether pre-conception health measures, such as iron, folate or vitamin deficiencies can be associated with obstetric complications during pregnancy; which are in turn associated with ASD.

Identification of defective placental function has become a focus of prenatal care, monitored by first-trimester serum markers such as Placental Protein 13 (Romero, et al., 2008), Placental Growth Factor (PIGF), and uterine artery Doppler ultrasound (Kuc et al., 2010; Myatt, 2010). Preeclampsia is characterized by oxidative stress and permanent systemic vasoconstriction (Aris et al., 2009; Myatt, 2010), and has been associated with metabolic-syndrome like metabolic effects which were indicated by elevated maternal serum fatty acid binding protein 4; FABP4 (Scifres et al., 2011). Maternal metabolic-syndrome like effects were indicated by elevated maternal serum fatty acid binding protein 4; FABP4 measured at 13 weeks gestation; before onset of preeclampsia (Scifres et al.). A similar lipid biomarker has been used to screen nonpregnant individuals with suspected dyslipidemia and insulin resistance. Lower midgestational serum vitamin D levels were associated with high odds, $OR= 3.63$, 95% CI[2.02-14.52] of severe preeclampsia (Baker, Haeri, Camargo, Espinola, & Stuebe, 2010) in a U.S. case-control study. Robinson, Alanis, Wagner, Hollis, and Johnson (2010) confirmed reduced serum vitamin D levels were associated with early onset, severe preeclampsia in a separate population.

Amin, Smith, and Wang (2011) summarized the results of 13 studies on the relationship of neonatal jaundice and ASD for *DSM-III*, *DSM-III-R*, *DSM-IV* and ICD-9

case definitions. Matched controls often included unaffected siblings, suggesting adjustment for genetic and other environmental factors. Only 6 of 13 studies adjusted for confounders such as birth weight, gestational age, Apgar score, birth order or infant gender. The formal meta-analysis showed despite high cohort heterogeneity and minimal publication bias, results indicated jaundice, assessed by total serum bilirubin was associated with ASD, $OR= 1.43$, 95% CI [1.22- 1.67] for studies involving at least 30 children. Jaundice was not consistently defined, but was estimated as serum bilirubin greater than 10 mg/dl for birth weight above 2500 gram (Amin et al., 2011). Croen, Yoshida, Odoul,i and Newman (2005) did not find an association of billlirubin levels as a measure of jaundice and ASD in a California neonatal study cohort.

Mechanisms. Swamy, Ostbyn, and Skjaerven (2008) conducted a 20-year longitudinal study of preterm and low birth weight infants and reported preterm birth rate was higher among boys, and that preterm infants had a increased risk of childhood mortality, were more susceptible to illness until the age of 10, were more prone to developmental and educational delay, disabilities and mental handicaps, and their adult rates of reproduction were lower. These results suggest preterm male infants were more adversely affected than girls with consequences for mortality and development disorder risk as well as biologic abnormalities. Froehlich-Santino et al. (2014) reported respiratory distress and hypoxia were associated with increased risk for ASD in males, $OR= 1.99$, 95% CI [1.04-3.80] whereas jaundice was associated with increased ASD risk in females, $OR= 2.94$, 95% CI [1.28-6.74] in a California twins study enrolled during the period of 1987-2004 in a Stanford University family cohort.

Maimburg, Bech, Vaeth, Moller-Madsen, and Olsen (2010) recently concluded that for a population-based cohort study of live, full-term births in Denmark between 1994 and 2004, neonatal jaundice was associated with increased (56-88%) hazard ratio of psychological disorders, including ASD. The excess risk of infantile autism was 67% higher if the child was conceived by a multiparous mother or was born between October and March. Infantile autism prevalence was higher for boys than girls born between October and March. A recent clarification of the *ICD* codes included in the term “jaundice” as used by Mainberg et al. was published and the hazard ratio (*HR*) was revised from an estimated 1.56 to 1.25; the researchers did not report analysis of bilirubin level or breastfeeding duration on the relationship to ASD (Rosti, Lambertini, Stucchi, & Condo, 2011).

In the AGRE cohort, Lee, Newschaffer, Lessler, Lee, Shah, and Zimmerman (2008) reported a trend toward higher ASD among singletons and multiple births born in April, June, and October. ASD concordant multiple births were higher in March, May, and September. The authors concluded non-heritable factors during the pre or perinatal period influenced ASD risk and gender differential ASD risk. The nongenetic, gender-based findings add to the body of evidence of a plausible relationship of prenatal health, liver dysfunction or immaturity and ASD diagnosis, particularly among boys.

Male embryos reportedly grow more slowly or at sporadic rates, whereas female embryos experience more constant growth hormone due to growth hormone-liver dimorphism (Steinman & Mankuta, 2013; Wauthier & Waxman, 2008). A sex-specific susceptibility threshold to fetal assault may be moderated by sex hormones (Baren-Cohen

et al., 2011; Field 2014; Hu et al., 2009; Ober et al., 2008). Similar theories of growth hormone or growth factor sex-differences, and male predominance (1.2- 1.6 per 1.0 female) at the time of fertilization have also been proposed to account for the male risk differential in childhood cancers (Dorak, Pearce, Hammal, McNally, & Parker, 2007).

In conclusion, preterm infants (less than 33 weeks) were reported to have an excessive risk of ASD. Placental dysfunction, resulting in preeclampsia was associated with fetal oxidative stress, and low gestational Vitamin D intake and maternal proteinuria. Infants born in the winter or early spring (Hendrix, 2011) and/or in Northern climates tend to be at higher risk for jaundice and autism- particularly among boys born to multiparous mothers. Gender differences in growth hormone, liver function and xenobiotic clearance and liver maturity were speculated to reduce neonatal infant male resistance to smoking exposure, and enhance risk of mitochondrial dysfunction and lipid oxidation in males.

Preeclampsia and other symptoms of placental restriction maybe associated with fetal distress, and hypoxia. Among infant and children, mitochondrial dysfunction and enhanced oxidative stress have been strongly associated with ASD (Abrahams & Geschwind, 2008; Hu et al., 2009; Taurines et al., 2010). Obstetric complications may be an initial trigger for biochemical dysfunction which present as metabolic dysfunction such as oxidative stress and hypoxia in later life (Burstyn et al., 2011; Mann et al., 2010). Krakowiak et al. (2012) and Langridge et al. (2013) reported maternal hypertension, diabetes, and obesity were associated with ASD but did not report gender-differential effects. Langridge et al. proposed preterm delivery may be related to infection or

inflammation during pregnancy associated with cytokine development and the potential impact on dendrocyte and myelin sheath development, or associated with placental dysfunctions, preeclampsia, and fetal growth restriction. There is substantial evidence that perinatal maternal health likely affected fetal programming epigenetics which affect gender-differentiated birth outcomes such as jaundice delivery, infant hypoxia, and preeclampsia, and ASD risk.

Perinatal Diet Effect on Birth Outcomes

Vitamin D. Placental transfer and placental dysfunction, maternal diet, fatty acid and fat soluble vitamin status (i.e. vitamin D) research are active research areas associated with "fetal programming" hypotheses (Baker, Haeri, Camargo, Espinola, & Stuebe, 2010; Novak & Innis, 2012; Robinson, Alanis, Wagner, Hollis, & Johnson, 2010; Saugstad, 2011). Parathyroid hormone (PTH) involvement in calcium homeostasis has been extensively studied over the past 25 years (Bergwitz & Juppner, 2010, McCann & Ames, 2008), but insight and understanding of the critical role of the hormonal bone-parathyroid-kidney axis, modulated by liver generated active vitamin D species and CYP gene expression reflects more recent research (Levenson & Figueiroa, 2008; Smolders, Moen, Damoiseaux, Huitinga, & Holmoy, 2011). There appear to be fundamental gender difference in serum vitamin D levels or metabolism among Caucasian men and women (Bolland et al., 2007; Cannell, 2008; Hagenau et al., 2009). Because of the renewed interest in adequacy of Vitamin D in the U.S. diet, the suggestive evidence of a relationship between vitamin D status with obstetric complications, and the relationship of maternal diet status and fetal development, studies cited in this section relate to effect

of gender-associated Vitamin D metabolism on birth outcomes such as small for gestational age, preeclampsia, jaundice, and liver function.

Several rodent models and fewer human studies suggest biological plausibility and some evidence of association between vitamin D inadequacy and offspring cognitive and behavioral performance (McCann & Ames, 2008). Vitamin D deficiency appears to permit and support expression of vitamin D receptor proteins (VDR) in developing fetal brains, but the VDR appeared to be non-functional or altered as a result of vitamin D deficiency (Haussler, Jurutka, Mizwicki, & Norman, 2011; Levenson & Figueiroa, 2008). Vitamin D receptor modulates several gene transcriptions which affect intestinal absorption, calcium, and skeletal homeostasis (Haussler et al.). Maternal vitamin D depletion affected fetal rat brain neuronal regulation and interrupted anti-inflammatory processes (Cui, McGrath, Burne, Mackay-Sim, & Eyles 2007; Eyles, Brown, Mackay-Sim, McGrath, & Feron, 2003; McCann & Ames, 2008).

The literature suggested a relationship between low serum 25-hydroxyvitamin D and risk of preeclampsia, and independent association with CYP gene expression; key factors which may affect birth outcomes. Low maternal vitamin D levels in the third trimester of pregnancy have also been associated with risk of subsequent autism (Dietert et al., 2011). No original research studies were identified which reported a direct relationship between prenatal vitamin D levels and gender-stratified autism diagnosis; perhaps because of the prolonged onset of autism diagnosis and the recall errors related to dietary recall, dietary nutritional, hormonal intake estimation, and associated measurement biases for hormonal regulation, various bioactive forms of vitamin D and

the impact of sunlight exposure on vitamin D bioavailability. There are several bioactive forms of vitamin D which are preferentially stored in various body tissue, enter circulation depending on health, nutrition and hormonal status and fluctuate rapidly but also vary seasonally (Cannell & Hollis, 2008; CDC, March 2011; Haussler et al., 2011; Heaney & Holick, 2011; Ross, Taylor, Yaktine, & Del Valle, 2011). Vitamin D biomarker research is an active area of research but may not provide adequate stability and external validation to be used as a biomarker in retrospective studies such as those designed to study the relationship to subsequent autism diagnosis. It seems plausible that a more robust measure of vitamin adequacy and vitamin D level may be related to vitamin supplement usage.

De-Regil, Palacios, Ansary, Kulier, and Pena-Rosas (2012) conducted a meta-analysis for the World Health Organization to determine whether vitamin D supplementation alone or in combination with calcium and other vitamin and minerals improved maternal and neonatal outcomes. Randomized and quasi-randomized Cochrane Pregnancy and Childbirth Groups Trial Register, the International Clinical Trials Registry Platform, and the Networked Digital Library of Theses and Dissertations were searched through October 2013. Interim results of six completed studies and 10 on-going studies suggested maternal serum vitamin D levels were higher among women using vitamin D supplementation, but highly heterogenous compared to pregnant women who received no intervention or placebo. Health outcomes related to preeclampsia and birthweight were inconclusively suggestive of benefits associated with vitamin D supplementation. Single dosage effect of supplementation showed no differences in the health outcomes of

stillbirths, neonatal death, or nephritic syndrome. No studies reported effects on preterm birth, maternal death, rate of admission to intensive care, or infant Apgar scores. The authors concluded inconclusive effects of single or continued vitamin D supplementation trials during pregnancy due to a lack of reproducible trial results.

Mechanisms. Researchers have suggested a fundamental gender difference in vitamin D serum 25 hydroxyvitamin D levels or metabolism exists among men and women and speculated the gender differential is perhaps related to estrogen in adults (Bolland et al., 2007; Cannell, 2008; Hagenau et al., 2009). It is unclear whether estrogenic protective effect may account for the higher plasma vitamin D levels in infant girls as reported by Ostergard et al. (2011). But fat-soluble vitamin levels may represent metabolic proxy measures for maternal diet adequacy, placental transport function, preeclampsia and jaundice risk as well as breast milk quality. These birth outcomes are known to be associated with autism, and the gender differential in ASD. Due to the genetic variability in vitamin D receptor function, the effect of skin color, sunlight exposure, nutritional supplementation and diversity of foods which may include vitamin D (such as dairy products, boney fish) the overall association of vitamin D levels to ASD is a complex relationship (Kocovska et al., 2012). The relationship of plasma fatty acid profiles and ASD may reflect metabolic dysfunction, fish intake, or fish oil supplementation (Wiest, 2007). A generalized profile of prenatal health which reflects prenatal maternal diet which accounts for fish intake, nutritional supplement use may be associated with obstetric complications, autism and may inform the gender-differential of

autism. The effect of maternal nutrition and dietary intake on epigenetics and temporality of ASD risk factors deserves further research attention.

Perinatal Diet Effect on Offspring Epigenetics

The understanding of the impact of prenatal, maternal nutrition on birth outcomes has been studied more extensively than the impact of prenatal nutrition on offspring epigenetics. Epigenetic changes are documented for maternal diet and behavioral factors associated with subsequent offspring autism diagnosis. Reproductive health and maternal gene-diet interactions are speculated to significantly affecting the quality of eggs and uterine environment (Burdge & Lillycrop, 2010; Gabory et al., 2009). Several studies have shown permanent and reversible effects of maternal diet inadequacies with evidence-based effects of low global energy, protein-restricted diet, low polyunsaturated fatty acid intake, low folic acid intake, on birth weight and later life disease onset (Barker & Osmond, 1986; Burdge & Lillycrop, 2010; Phillips et al., 2009; Gluckman & Hanson, 2004; Heijmans et al., 2008; Koletzko, Larque, & Demmelmair, 2007; Lane 2011; Lillycrop, 2011; Ryan, Keske, Hoffman, & Nelson, 2009). Lillycrop (2011) suggested maternal diet deficiencies may promote methylation and induce stable alterations associated with gene transcription as described earlier by Gabory et al. (2009). Fatty acid levels of EPA and DHA have been shown to affect genetic transcription factor regulation as well as gene expression (Novak & Innis, 2012). Epigenetic methylation changes in genes within placental tissue, in response to maternal smoking have been documented as well (Pedersen et al., 2013; Wilhelm-Benartzi, et al., 2012).

Nutrients. Schmidt et al. (2012) reported folic acid supplementation three months before pregnancy, and first trimester was associated with lower risks of ASD. Results suggested one-carbon methylation of folate and cysteine metabolism-related (MTHFR, COMT and CBS) gene expression was altered for particular gene alleles in pregnant women. Steenweg-de Graaff, et al. (2014) reported lower social responsiveness scores were associated with low prenatal maternal plasma folate levels at 13 weeks gestation. But maternal folate use after delivery was not associated with ASD in offspring, suggesting timing of folate exposure to fetal development was critical in the Dutch population study. Lyall, Schmidt, and Hertz-Picciotto (2014) suggest periconceptual folic acid supplementation illustrated evidence for the association of prenatal vitamin and nutrient supplements to offspring ASD risk.

A literature review for the search terms *pregnancy, nutrition, autism, or maternal protein intake and autism, prenatal diet and autism* yielded papers which discussed environmental or chemical prenatal exposure, prenatal stress, and low birthweight outcomes and offspring of young anorexic women. Two additional papers discussed the potential risk of iodine deficiency on thyroid function in pregnant women. Several researchers have shown dietary DHA and EPA regulate fat and glucose metabolism through epigenetics, and were associated with protein intake, rather than fat intake (Novak & Innis, 2012). It is documented that several nutrients are exchanged through maternal placenta, so it was of interest to conduct a literature search related to placental nutrient transport affecting offspring. Human placental function involves both secretion

and absorption via one- or two-way nutrient transport (Pedersen et al., 2014; Sibley, 2009).

Mechanisms. Sibley (2009) summarized recent human studies, and emerging topics and less understood aspects of placental transport. Sheep and rodent models are likely to show lower placental permeability and diffusion rates compared to human placental physiology across a wide range of nutrients including sugars, salts, amino acids, and sugar alcohols. Human placenta is a “two-way” transport system, sensing maternal metabolism changes, and responding to support fetal growth trajectories (Sibley) in a dynamic, responsive manner previously described as being similar to “fetal programming” (Pedersen et al., 2014).

Electrochemical gradients, and fixed pressure diffusion are the primary mechanisms of nutrient placental transport. (Fowden, Ward, Wooding, Forhead, & Constancia 2006; Sibley 2009). The electric potential gradient of 8 to 10 millivolts, between fetus and mother is reduced over gestational trimesters to zero at term birth (Sibley, 2009). Fetal growth control appears to be regulated by sodium, calcium, potassium, and amino acid transport gradients (amino acids require transporter proteins). During fetal growth restriction, changes in activity of transporter proteins used to exchange nutrients such as carry leucine, lysine, System A (alanine/glycine), taurine, glucose, ion minerals, and lipoprotein lipase have been reproducibly measured and validated (Cleal & Lewis, 2008). In rat models, maternal testosterone levels induced fetal growth restriction and down regulated amino acid placental transport (Sathishkumar, Elkins, Chinnathambi, Gao, Hankins, & Yallampalli (2011). Preeclampsia, maternal

hypertension, low protein maternal diet and jaundice have also been associated with placental surface area reduction and fetal growth restriction (Aris et al., 2009; Jansson et al., 2006; Myatt, 2010; Pedersen et al., 2014; Tiedje et al., 2008). Placental surface area was decreased and the exchange barrier has been documented to physically thicken during fetal growth restriction (Pederson et al., 2014; Sibley, 2009; Myatt, 2010).

Maternal nutrition and metabolic environment has also been shown to modulate human placental exchange as evidenced in studies of women with varying arm muscle area and body mass indices (Osmond, King, Brennecke, & Gude, 2001). Maternal leptin and insulin-like growth factors levels and function may be associated with fetal adaptation and epigenetic mechanisms affecting fetal growth trajectories (Sibley, 2009). Koletzko et al. (2007) studied placental transfer of long chain polyunsaturated fatty acids in humans using isotope labeling studies and reported an intake or dose-related gradient which correlated with fatty acid transport protein 4 (FATP 4) expression. However, no literature was identified which reported offspring gender-specific placental transport mechanisms, metabolomics or epigenetic traits in offspring. Research on placental transfer is an emerging research area, and the challenges associated with non-invasive biometrics of fetal development deserves more focus. Research to date suggested gene expression, allele forms, and methylation of genes within human placenta may affect epigenetic mechanisms associated with “fetal programming” and “multiliability threshold” concepts (Nijmeijer et al., 2010; Schmidt. et al., 2012; Wilhelm-Benartzi et al., 2012). A hierarchical framework to integrate epigenetic factors of ASD is lacking.

Biomarkers. Genetic based marker research was increasingly abundant in the literature and studies published since 2004 have begun to suggest genetic alleles or gene expression may be gender based. Genetic variability or threshold phenotypic expression theories which may explain gender-differentials in autism diagnosis were recently reviewed (Rivet & Matson, 2011). Specific allele forms of genes related to liver function were found to be risk factors for autism among offspring via in utero exposure to medication (Connors et al., 2008; Croen, Grether, Yoshida, Odouli, & Hendrick, 2011). Medical hypotheses have speculated on the possible protective effect of estrogen against autism among infant girls (Baren-Cohen et al., 2011; Field 2014; Hu et al., 2009; Ober et al., 2008; Pastural et al., 2009) which may be associated with vitamin D receptor protein allele forms (Deng, 2003; Pihl et al., 2010) or cholesterol/steroid metabolism (Field, 2014; Hu et al., 2009; King, 2011).

Human studies suggest an epigenetic interaction of endogenous prenatal metabolites, and hormone related biochemicals and autism risk (Dietert et al., 2011; Higashida et al., 2010; Hu et al., 2009; King, 2011; Lyall et al., 2014; McCanlies, et al., 2009). Others reported neonatal environmental risks are genetic allele specific (Cheslack-Postova et al., 2007; Nijmeijer et al., 2010) or reflect an interaction of gene allele and diet (Higashida et al., 2010; Lillycrop, 2011; Schmidt et al., 2012). Genetic profiles inform protein and metabolic pathways associated with ASD, which in turn are characterized by metabolites (Hu et al.). Therefore, well characterized familial genomics may provide complimentary information to study the effect of parental smoking, maternal nutrition,

preeclampsia, jaundice, lactation, and SIDS-like interrupted breathing patterns on ASD and the gender differential.

Genetic Inheritance: A Summary of AGRE Genetic Literature

There is speculative and growing evidence for gender and genetic allele diversity related to vitamin B and D metabolism, CYP liver enzyme function, and methylation of placental tissue in response to maternal smoking and diet (Bolland et al., 2007; Habek & Kovacevic, 2011; Hagenau et al., 2009; Nijmeijer et al., 2010; Ostergard et al., 2011; Petersen et al., 2014; Pihl et al., 2010; Schmidt et al., 2011; Thomas-Moya et al., 2006; Wilhelm-Benartzi et al., 2012). Therefore, the study of proxy variables for parental age, maternal health, nutrition, placental exposures, infant traits and gender, within a hierarchical framework, for a well-characterized infant diet (i.e. lactation) within a well-characterized genomic cohort may help to reduce study confounding and bias.

Despite intensive genetic candidate identification and more than 20 genome-wide association studies for autism, few studies have identified reproducible gene markers for autism or assessed the clinical utility of genetic assessments (Carayol et al., 2011). A sample frame or population cohort of well characterized maternal/paternal/sibling genetic information as fixed or defined traits was chosen to represent a stable cohort with data registries which included standardized characterization of medical records, biomarkers, DNA, and genetic material.

Autism Speaks' AGRE is an open-source, nonprofit DNA repository and family registry database of genotypic and phenotypic information and medical records that are available to autism researchers worldwide (Lajonchere, 2012). The research consortium

program began in 1997 for families who have two or more children on the autism spectrum. AGRE was originally founded by the advocacy group, Cure Autism Now (CAN) in 1997, AGRE is currently funded by the National Institute of Mental Health (NIMH), National and Autism Speaks, which merged with CAN in 2006 (Lajonchere, 2007). Referrals from clinical and medical professionals are the primary ascertainment method and there is no restriction to age, ethnicity, or socioeconomic status. As long as there are two affected family members, parity is not considered (Lajonchere 2010). Prior to 2007, over 2,000 families had participated in the program representing the 50 United States. The majority of the sample (75%) was Caucasian and non-Hispanic and 37% of families were from the Western U.S. coastal states and autism affected offspring were all born since 1992 (Cantor et al., 2007). The use of artificial reproductive technology has also been documented within the registry (Lajonchere, 2012).

Most AGRE research articles understandably focused on genetic biomarker research. However, medical records are available for parental and family history, and child medical records. Of the more than 230 literature articles referenced on the AGRE website and available through other literature search engines, approximately ten percent of studies focused on gender-differentiated risk factors and genetic profiles (Hall, Huerta, McAuliffe, & Farber, 2012; Lajonchere, 2010). These studies may inform the gender differential of autism. AGRE literature on the effects of variables such as parental smoking, parental age, maternal diet, obstetric complications, and metabolomics of ASD were also reviewed to determine the relationship of genetic traits with phenotypic outcomes. Gender-specific traits associated with calcium expression genes were

reproduced in several for AGRE cohort studies. Effects of parental age, preeclampsia, maternal albuminuria, xenobiotic (CYP gene) metabolism, and ASD were reported to be associated with particular genomic traits within the AGRE registry cohort. Hallmayer et al. (2011) reported that for monozygotic and dizygotic twin cohorts enrolled in a California cohort, 38% of ASD risk was associated with genetic heritability and 58% was associated with shared environmental factors in utero or early infancy, suggesting “fetal programming” is a plausible biomechanism of ASD. Hu et al. (2009) suggested cholesterol/steroid metabolism may be associated with gender differential risk of ASD, as a result of studying AGRE ASD cases and siblings. However, Hu et al. studied genome-wide associations among cases and controls rather than particular chromosomes.

Chromosome factors. Ten AGRE gender-stratified studies validated an association with autism for Chromosome 17 regions, particularly among males, with subsequent substantial evidence of gene mapping suggestive of gene markers associated with calcium expression or function. Chromosome 17 is associated with the location of sex hormone binding globulin, retinoic acid receptors, homeobox B gene cluster, and serotonin transporter genes (Gilbert, 1998). Yonan et al. (2003) suggested an association of autism with genetic markers on Chromosome 17 for a sibling-pair linkage study for 345 multiplex AGRE families in which the researchers reported a logarithm of the odds (LOD) score of 2.8 for Chromosome 17. LOD scores indicate the gene proximity correlation among gene single nucleotide polymorphisms (SNPs). A high and positive LOD score implies cluster or block-inherited transmission during meiosis, across generations (Khoury, Bedrosian, Gwinn, Higgins, Ioannidis, & Little, 2010, p 23). When

the cohort sample was stratified to include only affected males, the linkage signal (LOD) increased to 4.3; whereas when stratified for affected females, the LOD decreased to zero. Cantor et al. (2005) replicated these AGRE results using an independent sample of 109 affected AGRE sibling pairs and interpreted the results to suggest a sex-specific genetic pattern. Stone et al. (2004) reported sex-specific chromosome regions on chromosomes 17, 4q32 and 10q for subgroups (male only and female containing) sibling cohorts of autistic children but concluded significant group overlap in the DNA samples from 148 families in the AGRE family registry. Subsequent survey of the DNA region flanking the centromere on Chromosome 17 showed nominally significant single nucleotide polymorphisms (SNPs) associated with myosin (MYO1D, neuronal expression (ACCN1) and neuronal migration and proliferation (LASP1) among Caucasians for a sample of 333 AGRE parent/affected child trios (Stone, Merriman, Cantor, Geschwind, & Nelson, 2007). Serajee and Mahbubul (2009) compared Y chromosome haplotypes among 146 AGRE autism-affected males and 102 controls of European-American descent and reported the two most frequent haplotypes were equally distributed among autistics and controls, but Monte Carlo tests with Clump® software showed a significantly different distribution of haplotypes after 100,000 simulations. These selected papers provided conclusive evidence of male-specific autism risks associated with Chromosome 17 genes.

Strom (2010) utilized integrative genomic approaches to identify eleven 17q21 candidate genes including calcium signaling and ubiquitin enzyme enriched in probands associated with autism. Strom et al. (2010) suggested a relationship within male-only

probands for a calcium channel gene (CACNA1G) and autism diagnosis in an AGRE cohort of 284 parent/affected male trios.

Carayol et al. (2011) reported a sex-specific genetic score to identify at-risk cohort siblings using the population of Stone et al. (2004) for autism spectrum diagnosis based on ten candidate genes; three genes were common to both genders (PITX1, SLC25A12 and ATP2B2). Ten candidate genes were selected based on previous research suggesting PITX1, a key regulator of hormones with the pituitary-hypothalamic axis may be implicated (Phillippi et al., 2007), and genes EN2, SLC25A12 and ATP2B2 demonstrated to have predictive ability in a genetic-score based model (Carayol, Schellenberg, Tores, Hager, Ziegler, & Dawson 2010) and four other genes with statistical association replicated in at least one independent study (HOXA1, GRIK2, ITGB3, and CNTNAP2 as well as MARK1 and JARID2 gene; the latter of which was strongly associated with autism diagnosis for the Autism Genetic Resource Exchange (AGRE) repository. GRIK-2 encodes for a receptor of L-glutamate, CNTNAP2 contactin neurexin gene reportedly associated with regressive language skills in males (Alarcon, et al., 2008; Arking, et al., 2008). Subsequent research by Carayol et al. (2011) using 277 AGRE families and a replication sample of 406 Italian families, replicated the importance of ATP2-B2 genetic allele variants as sex-specific risk factors. Allele transmission of the gene which codes calcium-transporting ATPase2 which in turn extrudes calcium from the cytosol into extracellular space showed recessive transmission patterns. Associations among three ATP2-B2 polymorphisms and autism were reported only for males.

The studies above suggested possible associations between particular genetic alleles within AGRE cohorts and subsequent autism diagnosis, but most researchers generally report the small number of representative females with autism as a study limitation. Study factors such as small effect size, possibilities of SNP interactions, gene-environment interaction, and statistical confounding have lead researchers to adopt statistical methods to address these challenges. Multiple testing, multifactor dimensionality reduction and machine learning methods are general techniques adopted for the use of analyzing SNP data. Using the AGRE population data, Schwender, Bowers, Fallin, and Ruczinski (2011) modified ensemble learner methods such as LogicFS (logistic regression applied to bootstrap samples) for case-control designs to address case-parent trio data to study interactions of SNPs. Simulation studies for 461 case-parent trios indicated a three-way interaction for GLRX3 (glutaredoxin-3) gene on chromosome 10. Independent tests for 138 SNPs showed pair-wise significance with glutathione-related genes showing the largest estimated marginal effect size. The researchers cautioned the approach was best suited for descriptive hypothesis generation rather than hypothesis testing; but concluded the statistical approach should be able to detect interactions with odds ratios much less than two. Lu and Cantor (2012) reported that the use of joint association tests of SNPs to account for interaction of gene and other risk factors increased statistical power in a case-pseudo-control design when gender was used as an independent risk factor for 990 AGRE families. The approach yielded two associations which exceeded genome wide significance; Ryadine Receptor-2, implicated in calcium channel defects, and a uridine phosphorylase-2 gene, associated with

glycogenesis or glycogenolysis. The latter gene was shown to be over-transmitted in both male-only and female-containing families within the AGRE population.

Gender differentiated factors. Heritability estimates have been studied to summarize the resemblance of offspring and parents by measuring phenotype variance attributable to genetic variance (Pan, Ober, & Abney, 2007). Estimated heritability allows partitioning observed variation in factors into unobserved genetic and environmental factors (Visscher et al., 2008). Narrow sense heritability reflects additive variance of phenotypic variance; broad sense heritability measures the proportion of all genetic variance (ie. additive, dominance, and epistatic effects); but both types have generally assumed equal genetic variance by gender (Abney, McPeck, & Ober, 2001; Moskau, Golla, Grothe, Boes, Pohl, & Klockgether, 2005).

No gender-stratified differences were reported for AGRE cohorts in studies related to obstetric variables and xenobiotic clearance associated genetic alleles. No gender differences were reported among 444 subjects from 228 AGRE families recruited for a study of the relationship of obstetric and psychiatric variables as predictors of autism severity using a nested linear mixed effect model (Wallace et al., 2008). The researchers reported maternal hypertension and edema were associated ($p < 0.01$) with higher ADI-R communication deficit and repetitive behavior scores. Preeclampsia was also associated ($p = 0.02$) with higher ADI-R communication deficit scores. Maternal albuminuria was associated ($p = 0.039$) with higher ADI-R repetitive scores as was parental depression ($p = 0.005$). Parental psychiatric variables of depression and anxiety

were also associated with higher ADI-R repetitive behavior scores, and lower communication composite scores.

Serajee, Nabi, Zhong, and Huq (2004) reported generalized (non-gender specific) associations for xenobiotic metabolism-related CYP gene polymorphisms and autism diagnosis for 196 AGRE trios using family-based association analysis and *chi-squared* analysis. The researchers studied expected transmission patterns for a metal-regulatory transcription factor (MTF1), an organic anion transporter (ABCC1), divalent metal ion transporters (SLC11A2 and SLC11A3), paraoxonase-1 (PON1), and glutathione S-transferase (GSTP1). Results showed deviations from expected patterns for SLC11A3 and MTF1 among ASD-positive subjects; no gender-differentiated analysis was reported.

The AGRE registry was established to focus on genetic inheritance factors of ASD. Therefore, due to the recruitment and ascertainment methods used in the AGRE registry, and a focus on genome wide association tests, paternal age has typically not been a variable of focus. However, Cantor et al. (2007) utilized data from an external U.S. reference group of 2.5 million non-twin births occurring between 1995-2000 to multiparous, Caucasian, nonHispanic, married mothers less than 36 years of age from all states (excluding California) to compare paternal age categories with autism diagnosis using chi-squared group statistics. The AGRE sample included 312 families, and paternal age groups of age 20-29, 30-39, and 40-49 years of age for mothers less than 36 years old at singleton first child birth. The researchers concluded the paternal age distribution of AGRE fathers differed significantly from the control sample and there was a shift toward higher paternal age in those with an ASD-affected first born child. The findings of Cantor

et al. (2007) regarding risk of increased paternal age associated with ASD were replicated by Anello et al. (2009) who used an AGRE cohort. The effect of birth order on ASD was described earlier (Lee et al., 2008; Maimburg et al., 2008). Such evidence has been replicated in the AGRE population (Martin & Horriat, 2012).

Martin and Horriat (2012) studied ASD symptom severity across birth order in an AGRE cohort as an indication of shared environmental factors which may affect ASD etiology. ASD severity was measured by verbal and non-verbal cognitive and repetitive behavior tests for 346 sibling-pairs. They reported first affected children had greater severity of ASD symptoms. Martin and Horriat replicated the findings of Durkin et al. (2008) who studied the CDC ADDM Network cohort which represents 18 states and metropolitan areas in the United States.

Studies described in this section suggested the AGRE registry may be representative of other U.S. cohorts of ASD children. Reproducibility of results with previous AGRE researchers was presumed to suggest adequate internal and external study validity (Carayol et al., 2011; Stone et al., 2004; Yonan et al., 2003).

AGRE demographic and socioeconomic characteristics associated with parental smoking and breastfeeding duration, for example represented the broader U.S. population prevalence for these behaviors (Gregory et al., 2013; Kalbrenner et al., 2012; Schultz et al., 2006; Shamberger, 2011). Therefore it was assumed the AGRE registry was a representative source of archival ASD data.

The use of AGRE medical records provided retrospective analysis of patient record histories, complimented by genetic inheritance, shared environmental, familial,

preconception, maternal, prenatal, and infant trait risk factors associated with ASD.

Shared environmental factors such as parental age and household smoke exposure may increase external validity of the study design compared to birth record datasets. AGRE access to multiple variables of preconception health, obstetric, parental smoking, and maternal diet measures may increase study validity, and reproducibility; and allow for the identification and interpretation of variable interactions. Evidence of risk factors such as increasing parental age, perinatal maternal health, preeclampsia, calcium and xenobiotic metabolism dysfunction have been replicated within the AGRE sampling frame.

Suggestive evidence for cholesterol/steroid and calcium-related metabolic differences in the AGRE registry has been identified using genomic association tests. These potential metabolic differences may inform the gender differential of ASD. There was a need to revisit the AGRE data collection to determine whether clinical record data variables may inform the suggestive gender-differential trends identified by several AGRE genetics researchers. There was opportunity to conduct ASD etiology studies which include gender-stratification to inform weak associations among complex gene-environment interactions. The relationships of these temporal exposures informed plausible multi-liability thresholds, placental transfer, or fetal programming which may explain ASD and the ASD gender differential.

Relationship of Prior Literature to the Study Framework

There are substantial gaps in the medical and epidemiological literature regarding mechanisms of ASD onset. The relative contribution of gender-associated prenatal and fetal exposure factors to autism diagnosis is unknown, despite decades of biochemical

epidemiologic research. The proposed conceptual framework, illustrated in Figure 2 attempted to address temporality of risk factors which may be associated with genetic susceptibility, epigenetic mechanisms, multiliability threshold, placental transport, or fetal programming mechanisms. Exposure-timing factors represented hypothetical preexisting genetic susceptibility (distal factors), direct placental exchange factors (main effect), and confounding genetic factors.

Table 3 summarized key studies in the literature that were aligned with the hypothesized main effect factors, preconception distal factors, and confounding factors. This AGRE study presumed exposures during pregnancy were key, predictive factors. The focus of this section was to summarize the relative risk and magnitude of effect of the hypothesized, temporally clustered preconception, prenatal, maternal, and infant trait risk factors associated with ASD and the ASD gender-risk.

Odds Ratio and Relative Risk Trends

While specific criteria and approaches have been published for conducting formal meta-analysis of ASD case control studies (Brasic & Holland, 2007; Kolevzon et al., 2007; Gardener et al., 2011; Guinchat et al., 2012), the purpose of Table 3 was to summarize overall effect measures for hypothesized main effect, distal, and confounding factors studied in the AGRE cohort. The main effect factors in Table 3 reflected an overall average effect estimate of 2.2 for maternal smoking, prenatal vitamin use, and lactation. The data for maternal smoking measured odds of ASD in offspring for mothers who smoked during pregnancy with an average $OR= 1.76$. Maternal vitamin use during pregnancy odds ratio associated with subsequent ASD risk ranged from nonsignificant to

significant values. Only one study reported odds of ASD risk among mothers who recalled breast or bottle feeding practice.

Quantitative measures of gender-based metabolites were included in the first page of Table 3 as references for the estimates of adequate sample size calculations to study ASD gender-risks. These quantified estimates were necessary to estimate power analysis for this dissertation. Some studies reported quantified parental smoke exposure effects (i.e. > 10 cigarettes/day) associated with SIDS risk (Habek & Kovacevic, 2011), but no literature was identified in the literature for the relationship of continuous smoke frequency to ASD.

Overall effect estimate of parental age, and preconception maternal health was 1.8 for studies on the second page of Table 3 for ASD and PPD outcomes (Krakowiak et al., 2012). The average, adjusted effect estimate of parental age was 2.0 for the 17 study comparisons shown in Table 3. In these studies, parental age was often categorized in 5 to 10 year increments. Overall effect estimate of hypothesized confounding variables of infant hypoxia, fetal distress and obstetric factors (excluding maternal fever and maternal drug use during pregnancy data) was 1.25. Obstetric health factors showed an average effect size of 1.4 whereas induced labor and hypoxia/fetal stress comparisons showed lower overall aggregate effect ratios (1.22, 1.1). Jaundice birth and cesarean-section delivery had higher *OR* ranges than induced labor.

Table 3

Potential Epigenetic Risk Factors of ASD and the ASD Gender Differential

Risk variable	Variable value	Risk metric 95% CI	Case count	Researcher and study population
Maternal Smoking	Binary response	<i>RR</i> =	3,315 PPD	Kalkbrenner et al., 2012 United States
	During pregnancy	1.26 [0.91 – 1.75]	633,989	
	During pregnancy	0.86 [0.79 – 0.93]	10,625 cases	Gregory et al., 2013 United States/NC
Maternal Health	Smoking 4.8 cig/day	<i>OR</i> =	645 ASD	Grazuleiciene et al., 2009 Lithuania
	Smoke & IUG-restricted	1.57 [0.45 – 5.55]		
	Smoke & GST allele	3.33 [0.60 – 18.4]		
Maternal Health	Binary Response	<i>OR</i> =	207 to 439	Nijmeijer et al., 2010 Netherlands
	Smoking & LBW Smoke & COMT & SLC6A4 alleles	~ 1.0 ~ 5- 6		
Prenatal Vitamin use	3 mo, 1 st trimester	<i>OR</i> =	429 cases	Schmidt et al., 2011 CHARGE study/CA
	Use (yes/no)	0.62 [0.40 - 0.93]		
	Use- yes and Infant COMT allele	1.80 [0.99 - 3.50]		
	Use-yes and Maternal MTHFR & CBS allele	2.60 [1.20 - 5.40]		
Maternal Diet	Vitamin D use in pregnancy		10 studies	DeRegil et al., 2012
	Binary Obstetric Outcomes			
	Preeclampsia	slightly suggestive effect on reducing preeclampsia risk		
	Birth weight	slightly suggestive effect on increasing birth weight		
	Single dose effect	inconclusive on still-birth, neonate death, nephritic symptom		
Maternal Diet	Ecological WIC study		prevalence	Shamberger, 2011
	Exclusively breastfed associated with lower thiamin, riboflavin, and Vitamin D			
Breastfeeding	None or Formula	<i>OR</i> =	861 cases	Schultz et al., 2006 AIRS-Internet study
	None for 6 months	2.48 [1.42 - 4.58]		
	Formula (no DHA)	4.41 [1.24 - 15.7]	924 cases	Dodds et al., 2011 Nova Scotia
	Initiation in hospital	1.20 [1.04 - 1.40]		
Child Plasma Vitamin D	Cases in Egypt	28.5 ng/ml plasma	112 cases	Meguid et al., 2010
	Controls/Typical	40.1 ng/ml plasma		
Child Plasma Vitamin D	Cases in US	20 ng/ml plasma	89 cases	Molloy et al., 2010
	Controls/(healthy?)	17 ng/ml plasma		
Gender effect Leptin & ASD	Plasma leptin in girls	mean = 2.11 ng/ml	80 cases	Ashwood et al., 2008 CHARGE study/CA
	Plasma leptin in boys	mean = 0.96 ng/ml		
	Leptin in ASD pos.	mean = 1.19 ng/ml		
	Leptin in PPD pos.	mean = 0.88 ng/ml		
Gender effect Metabolites	Females aspartic acid	> 10 nmol/l urine	34 cases	Evans et al., 2008 United States
	Females glucose	> 500 nmol/l urine		
Gender effect Fatty Acids	ASD & Female cases measured fish intake but not dietary supplements/fish oil	lower plasma DHA	153 cases	Wiest et al., 2009 Wiest 2007, U.S.

Risk variable	Variable value	Risk metric	95% C I	Case count	Researcher and study population
Parental Age	Mom & Dad	OR=		164 cases	Lauritsen et al., 2005
	Maternal 12-19 yrs	1.7		11,000 births	Sweden population
	Maternal 25-29 yrs	1.1			
	Maternal > 30 yrs	1.2			
	Paternal < 25 yrs	2.5 unadjusted., 1.9 after adjustment for zygosity and SES			
	Paternal > 50 yrs	3.2 unadjusted., 3.4 after adjustment for zygosity and SES			
	Maternal age (</> 35)	1.6	[1.32 - 1.95]	64 studies	Gardener et al., 2011 multiple cohorts
	Paternal age (<30,>40)	3.1	[0.95 - 9.49]		
	Paternal age(<30,>35)	1.3	[1.16 - 1.54]		
	Maternal age delta	1.3	(26 vs 32 years)	10,625 cases	Gregory et al., 2013 United States/NC
Parental Age	Mom & Dad	OR =		23,311 PPD	Grether et al., 2009 United States/CA
	Mom 10 yr incr.	1.38			
	Parental 10 yr incr.	1.3			
Parental Age	Mom & Dad	OR=		1,251 cases in 326,785	Durkin et al., 2008 US-CDC/ADDM
	Mom age >/< 35 yrs	1.3	[1.1 - 1.6]		
	Dad age >/< 40 yrs	1.4	[1.1 - 1.8]		
Paternal Age	10 yr intervals. on ASD	OR =		593 cases in 132,844	Croen et. al., 2007 United States/CA
	10 yr intervals. on Girls	1.55	[0.93 - 2.59]		
	10 yr intervals. on Boys	1.27	[1.01 - 1.60]		
ASD Gender Differential	Parental Age	OR=		393 cases	Anello et al., 2009 AGRE registry
	Dad age < 30 yrs	6.2			
	Dad age 40-44 yrs	3.3			
Preconception Health	Aggregate Response (Diabetes+ Obesity+)	OR=		517 ASD	Krakowiak et al., 2012 CHARGE study /CA
	(Diabetes+Obesity+ & Hypertension+)	2.35	[1.43 - 3.88]	172 DD	
Preconception Health	Pulmonary, heart, renal, anemia disease	1.25	[1.01 - 1.55]	924 cases	Dodds et al. 2011 Nova Scotia
Preconception Health	Diabetes	1.24	[1.08 - 1.42]	10,625 cases	Gregory et al., 2013 United States/NC
	Hypertension	0.94	[0.84 - 1.05]		
Maternal Health	Binary Response	OR=		691 cases	Mann et al., 2010 United States/SC
	Prenatal Smoking (+)	1.02	[0.80 - 1.30]		
	Preeclampsia (+)	1.85	[1.38 - 2.47]		
Maternal Health	Prepregnancy weight	1.58	[1.26 - 1.98]	924 cases	Dodds et al. ,2011 Nova Scotia

Risk variable	Variable value	Risk metric	95% C I	Case count	Researcher and study population
Infant Hypoxia	Full term males	<i>OR</i> = 1.11	[1.0 – 1.6]	113 cases 17,083 tested in 218,890 adjusted for birth year and SES	Burstyn et al., 2011 Canada
	Males tested at birth	1.13	[0.96 - 1.33]		
	Full term females	0.92	[0.60 - 1.42]		
	Females tested	0.93	[0.62 - 1.40]		
Delivery Event	Fetal stress	1.24	[1.08 - 1.42]	10,625 cases	Gregory et al., 2013 United States/NC
Delivery Event	Induced labor	1.22	[1.03 - 1.44]	924 cases	Dodds et al., 2011 Nova Scotia
Delivery Event	Induced labor	1.22	[1.01 - 1.46]	10,625 cases	Gregory et al., 2013 United States/NC
Obstetric Health	Intrauterine Growth IUG restr & LBW Placental methylation CpG analysis & LBW	<i>OR</i> = > 1.0 > 1.0 > 1.0 with effect of maternal smoking/drinking		380	Wilhelm-Benartzi et al., 2012. United States
Obstetric Health	At birthwt > 2500 g High bilirubin	<i>OR</i> = 1.43	[1.22 – 1.67]	13 studies 30 cases each	Amin et al., 2011 multiple cohorts
Obstetric Health	Adj. for confounders	<i>OR</i> = 1.56**	[1.05 – 2.30]	1,721 PPD 35,766 tested per 733,826 records for parious (nonfirst birth) mothers	Maimburg et al., 2010 Denmark
	Jaundice- binary (+/-)	1.88	[1.17 – 2.71]		
	Jaundice- binary (+/-)				
Obstetric Health	Mixed Effect Modeling			1,179 ASD (451 ASD without DD)	Langridge et al., 2013 Australia
	Elective C-section	1.44	[1.03 – 2.02]		
	Emergency C-section	1.47	[1.05 – 2.06]		
Maternal Fever during Pregnancy	Untreated fever	<i>OR</i> = 2.55	[1.30 – 4.99]	538 cases	Zerbo et al., 2013 CHARGE study United States/CA
	Treated fever	1.30	[0.59 - 2.84]		
Maternal Health during pregnancy	First trimester SSRI use	<i>OR</i> = 3.8	[1.8 – 7.8]	298 cases	Croen et al., 2011 CHARGE STUDY United States/CA

Note. cig= cigarette; PPD = pervasive developmental disorders; IUG = intra-uterine growth; GST = glutathione transferase; COMT = catechol-O-methyltransferase; SLC6A4 = serotonergic neurotransmission gene; MTHFR = methylene-tetrahydrofolate reductase; CBS = cysteine vitamin B-mediated sulfuration enzyme; DHA= decahexanoic acid; SES = socioeconomic status; , LBW = low birth weight; Adj.= adjusted; CpG = cytosine-phosphodiester guanine genomic regions; C-section = cesarean-section delivery; DD= developmental disorders; SSR I= selective serotonin reuptake inhibitor.

** Maimburg et al., 2010 odds estimate revised downward to 1.25 (Rosti, Lambertini, Stucchi, & Condo 2011)

Main effect variables. With regard to specific, independent variables and detailed comparison in Table 3, genetic factors appear to reflect larger effect estimates, or preexisting pregnancy complications. The data suggested odds ratios and relative risks tended to be greater than 2.0 for the risk variables mediated by particular genetic alleles, maternal untreated fever during pregnancy, prenatal serotonin regulation using SSRI medication, lack of breastfeeding, or infant formula use without docosahexaenoic acid (DHA) fortification, and increased paternal age. These particular factors may suggest a predominant effect of genetic variation or maternal infection or disease. The high odds ratio for breastfeeding in the Schultz et al. (2006) study may reflect the study sampling design (convenience, snowball, and internet solicitation).

Prenatal maternal health as a risk factor associated with ASD was highest for maternal serotonin-regulation via anti-depressant use and untreated maternal fever during pregnancy as shown in Table 3. However, several researchers suggest maternal diabetes; hypertension or pre-existing medical conditions were independent risk factors of ASD (Dodds et al., 2011; Gregory et al., 2013; Krakowiak et al., 2012). The prevalence of pregnant women with diagnosed diabetes or hypertension was less than 1.5%; but approximately 7.6% of pregnant women had gestational diabetes in a California cohort of women sampled from 1999 to 2005 (Lawrence et al., 2008). Maternal health status during pregnancy needed to be studied further in detail.

The effect of prenatal smoking showed an average odds ratio of 1.2 for four studies illustrated in Table 3. Measurement and recall bias was known to be a particular concern among self-reported measures of maternal smoking (Dietz et al., 2011;

Kalkbrenner et al., 2012; Lee et al., 2012; Burstyn et al., 2012; Vinikoor et al., 2010; Zhang et al., 2010). Because of risks of self-reported bias and rarity of self-reported prenatal smoking among mothers of autistic children, additional proxy variables suggestive of smoking behavior (i.e. paternal smoking, paternal and maternal age) and multiple proxy variables of maternal health may be useful (Dodds et al., 2011). Self-reported household smoke exposure may be a valid proxy variable; particularly for data collected separately, for both parents.

Self-reported maternal smoking which may be confounded by socioeconomic factors, was shown to be associated with certain serotonin alleles, and adverse birth outcomes such as placental restriction, preeclampsia, infant hypoxia, and ASD (McDonald et al., 2006; Nijmiejer et al., 2010; Wilhelm-Benartzi et al., 2012). Smokers may have reduced peroxidase activity in which to offset tissue lipid oxidation (Wang-Sattler et al., 2008). Transmission of cotinine has been documented in breast milk and infant's first meconium (Onuki et al., 2003; Gray et al., 2010). Therefore, direct gas or fluid exchange mechanisms may significantly affect ASD risk. A large U.S. cross-sectional study showed a trend toward inadequate infant nutritional status for infants who were exclusively breastfed (Shamberger, 2011). Infants with low DHA lipid intake may have higher risks of ASD (Schultz et al., 2006). Maternal dietary intake, smoking status, and breastfeeding duration were cited as main effect factors for ASD in at least three studies (Mann et al., 2010; Ronald et al., 2010; Schultz et al., 2006). There may be an ASD risk effect associated with gender-specific vitamin D mechanisms or fatty acid metabolism (Baker et al., 2010; Hu et al., 2009; Robinson et al., 2010; Weist et al.,

2009). Preeclampsia was shown to have a larger effect than smoking on ASD, but birth weight mediated the effect in one study (Mann et al., 2010). Exposure-timing research of smoking and obstetric traits is needed.

Distal variables. In Table 3, the odds ratio of preeclampsia, cesarean section delivery, jaundice birth associated with ASD diagnosis in offspring ranged from 1.25 to 1.9. There are likely multiple factors affecting obstetric complications, including preconception health. Placental health proxy variables such as preeclampsia, jaundice, and hypoxia may have a gender-specific effect on autism (Burstyn et al., 2011; Maimburg et al., 2010; Mann et al., 2010). The effect of these factors, stratified for assisted reproductive technology use on ASD risk, and the ASD gender differential are research gaps. And few ASD studies in the literature controlled for ART use.

The relationship between infant health and ASD is often reportedly confounded by infant traits such as gestational age, birth weight, Apgar score, infant gender, birth order, obstetric complications, or child IQ (Amin et al., 2011; Dodds et al., 2011; Gardener et al., 2011; Langridge et al., 2013; Mann et al., 2010; Schnedel & Bhasin, 2008; Wilhelm-Benartzi et al., 2012). There are likely interrelated effects of obstetric complications and neonatal infant traits such as breathing and sleeping patterns. Neonatal respiratory distress, weak or no crying after birth, oxygen treatment or resuscitation, infant fever, breathing; and feeding difficulties and infant anemia, respectively were among 13 of 60 obstetric risks associated with ASD (summary effect estimate, *OR or RR*= 1.7 – 1.85 for infant breathing and distress; 3.3 – 7.87 for feeding difficulties and anemia) in a meta-analysis of 46 global case-control studies (Gardener et al., 2011).

Dodds et al. (2011) explored several independent ASD risk factors; and discussed the strengths and weaknesses of using overall obstetric "optimality" indices versus individual obstetric and neonatal factors in a case-control study design to account for inter-related delivery and infant traits. In their sampling frame of 129,733 infants born between 1990 and 2002 in Nova Scotia, among 924 ASD cases, the effect of overall optimality indices and individual obstetric factors was non-significant in the association with ASD diagnosis. Among cases with low genetic susceptibility, the effect of infant gender, maternal pre-pregnancy weight, maternal health (pulmonary, heart disease, renal disease, and anemia), lack of delivery labor, and income support during birth year or first two years were shown to have an independent role in ASD etiology. The study publication did not discern whether maternal health conditions were diagnosed before or during pregnancy. But the study provided evidence that maternal health was an independent risk factor of ASD. Breastfeeding initiation during hospitalization was an additional independent risk factor, $OR= 1.20$, 95% CI [1.04 - 1.40] in the final multivariate model. Lactation capacity and duration is likely affected by maternal diet and health status.

Dodds et al. (2011) did not discuss whether the use of preconception ART was controlled or stratified in the case-control study. Similarly, the Canadian population case-control cohort results of Burstyn et al. (2011) and U.S. cohort case-control cohort studies of Mann et al. (2010), CHARGE studies shown in Table 3, and the study of Gregory et al. (2013) did not discuss whether ART factors were considered. A recent assessment of U.S. ART prevalence based on federally mandated fertility clinic certification records,

vital statistics, and CDC pregnancy monitoring systems suggested the use of ART by women over the age of 40 was between 16.5% and 27.9% in Florida, Utah, and Maryland in 2004 (Barradas, Barfield, Wright, D'Angelo, Manning, & Schieve, 2012). In a case-control design within an ASD registry for Israeli participants enrolled from 1995 to 2002, of participants diagnosed with ASD, 10.7% were conceived by ART, whereas the rate of ART pregnancies in the Israeli newborn cohort was 3.06% for infants born within the same period (Zachor & Itzchak, 2011). The study of ART on the association of pregnancy factors and ASD deserves attention.

Confounding variables. The hypothesized main effect, independent risk factor effect estimates for ASD summarized in Table 3 suggest prenatal health (diabetes, heart, renal disease, anemia), parental smoking, and lactation appear to be statistically significant, despite mediating effects of distal and confounding factors. Distal factors such as parental age, and pre-existing maternal health conditions, and obstetric complications appear to mediate the effect of pregnancy-related factors on the relationship to ASD, and the ASD gender-differential.

Table 3 illustrated substantial evidence that parental age affects ASD risk profiles, and the gender differential of ASD. It is hypothesized that parental age reflects a key genetic risk. Younger parental age appeared to reflect increased risk behavior such as smoking and alcohol intake whereas older parental age may have reflected chromosomal and cellular damage risks. Neonatal infant traits, such as infant sleeping and breathing patterns were theorized to reflect extraneous, or unique mechanisms such as respiratory infections, congenital heart defects, or side effects of psychotherapeutic medicines.

Common gender-associated profiles for hypoxia, other PPDS, sudden infant death and ASD have been reported. SIDS risk may also be congenital heart defect risk (American Academy of Pediatrics, 2011), an X-chromosome related risk (Baillard & Anderson, 2009; Gioli-Perira et al., 2008) or associated with cosleeping (Vennemann et al., 2005).

It was reasonable to hypothesize there may be an association for proxy measures of direct cellular, metabolic, gas or fluid exchange between a pregnant woman and a fetus (either through placental transfer and/or lactation) and subsequent ASD onset. Thus, maternal diet proxy biomarkers for exposures during pregnancy such as boney fish intake, vitamin supplementation, maternal smoke exposure and lactation duration may be significantly associated with ASD ; with or without gender stratification (James, 2012; Kalkbrenner et al., 2012; Kocovska et al., 2012; Lee et al., 2011; Shamberger, 2011; Wiest et al., 2009; Zhang et al., 2010). Placental transfer was theorized to reflect ASD epigenetic mechanisms.

There appeared to be an opportunity to assess genetically susceptible, maternal health risk factors which may be present *before* conception (i.e. anemia, vitamin deficiency, diabetes, hypertension, neural tube defect risk, and albuminuria) as unique and separate proxy measures. The contribution of assisted reproductive technology would likely be a distal factor, too. Preconception factors and ART were hypothesized to reflect genetic susceptibility; a different etiological pathway than pregnancy onset, and maternal health factors associated with more direct placental exchange *during* pregnancy or biochemical transfer via lactation practice. However, a biologically plausible overall index of genetic susceptibility or preconception maternal health index

may improve statistical power of the study to assess the relationship of other proxy variables on the gender differential of autism. Infant gender was presumed to be a significant main effect factor associated with ASD given the body of evidence in the etiology literature. However, infant gender is likely a confounder variable given the known ASD gender-risk.

Few previous cross-sectional or case-control studies on maternal smoking, maternal health status or lactation duration adjusted for pre- or postnatal nutritional fortification. Most previous studies did not address the gender differential of ASD. Gender differences in ASD cases have been identified in leptin adipose tissue hormone, amino acid, and glucose urinary samples, and triglyceride profiles (Ashwood et al., 2008; Evans et al., 2008; Wiest et al., 2009). However, these studies did not ascertain the effect of maternal diet, maternal or child vitamin use, placental health, obstetric complications, or lactation duration on childhood metabolic profiles. Most ASD related studies have not accounted for pharmacotherapy use by subjects. Temporal clustering of exposure-timing risk factors as genetically susceptible preconception factors, onset pregnancy with associated fetal exposures, and neonatal traits may provide insight into the relationship of indefinite windows of exposures and ASD, and the gender risk of ASD.

Etiological Hierarchy Framework

A hierarchical framework of ASD causal mechanisms may explain potential relationships among preconception, environmental exposures, affected epigenetic and biochemical pathways, and, metabolic processes in a fetus or infant. Biological plausibility was assumed in the proposed framework using antecedent exposures to

inform exposure-timing risks. Plausible mechanisms of fetal programming and placental transfer were assumed. Dodds et al. (2011) speculated leptin may play a role in placental transport dysfunction. However, Ashwood et al. (2008) reported leptin levels were highest among females regardless of ASD status (see Table 2).

Vague hypotheses have attempted to explain the observation of trebled or quadrupled ASD prevalence ratio of prepubescent males to females (Faber, Zinn, Kern, & Kingston, 2009; Hu et al., 2009; Pastural et al., 2009). But few studies were able to achieve statistical power to study the gender-differential of ASD in a case-control design. AGRE genetic research has provided suggestive evidence genetic and epigenetic for the main effect risk factors of ASD, and gender-differential of autism. Independent AGRE cohort studies have supported published evidence in other ASD based population cohorts within the U.S., and within other population sampling frames. However, few AGRE studies focused on the etiological hypothesis of genetic versus epigenetic mechanisms, or temporal factors associated with preconception, fetal exposures or neonatal traits affecting ASD and the gender-differential of ASD.

There was speculative comment in the literature that estrogen or growth hormone related mechanisms may provide protective effects against adverse health risks for females in the womb and at neonatal stage (Faber et al., 2009; Hu et al., 2009; Pastural et al., 2009). It was difficult to identify research papers which proposed mechanisms and simultaneously quantified related biomarker associations for the gender-differential in autistic children. Few publications have definitively ascertained parental or childhood genetic profiles, hormonal or quantified metabolic profile differences for gender-stratified

cohorts due to the ethical considerations. Collection of biological tissue and repetitive testing conducted during pregnancy would be unacceptably invasive and may be unethical. Genetic gender embryo testing, and genetic screening tests are not recommended or promoted for predicting ASD onset. The rarity and broad-based spectrum of autism symptoms (particularly among girls), prolonged onset of ASD and PPD, and reversible nature of PPDs are other ethical considerations for ASD gender-based testing. Limited clinical sample sizes, control of confounding variables, genomic variation, nonbiased sampling, and robust study designs constrained ASD gender differential research. Well-controlled studies within a specific genome and defined exposures were needed.

The AGRE repository provided an open access database of enrollment records for a well-characterized genomic cohort. AGRE literature was representative of other U.S. cohort findings and reported suggestive evidence of biological plausibility with results of other ASD study cohorts. AGRE genomic profiling may inform future studies with regard to the mediating effect of genetic alleles on prenatal and infant exposure risks, and inform the ASD gender differential.

The research questions in this study addressed the exposure-timing relationship of unique pregnancy factors to ASD outcome; and the effect modification (separately and in combination) of preconception and neonatal factors to ASD diagnosis, and the gender differential of ASD. The study proposed sought to describe and study the relationship of maternal smoke exposure and diet status during pregnancy, and lactation (adjusted for covariates and neonatal traits) to subsequent autism diagnosis with and without infant

gender stratification. For simplicity, the overall logistic regression hypothesis among the nine independent variables can be described as: "What are the exposure-time relationships between levels of maternal smoke exposure and maternal diet factors during pregnancy and lactation to ASD outcome when adjusted for preconception factors, including obstetric complications and confounding infant traits?"

The study aimed to replicate previous research on the relationship of parental age, smoking status, and infant gender to ASD. The study contributed to the limited body of research related to the proposed effect relationship of maternal smoke exposure and diet during pregnancy, and lactation to ASD and the ASD gender differential. The third study aim was to study the effect modification of preconception and neonatal factors on the pregnancy-ASD relationship. The study acknowledged effects of preconception health, ART use, and possible gender-specific exposure-timing relationships to ASD and the gender differential of ASD.

Family history records within the Autism Genetic Resource Exchange (AGRE) repository supplement coded individual, gender, parental, and familial characterization of genetic marker data. The archived parental and child record history files within the registry data set were collated and extracted to select variables to test the hypothesized conceptual framework presented in Figures 1 and 2. Standardized survey questionnaires were the source documents for data collection of variables defined in the conceptual framework of hypothesized main effect, distal correlates, and confounding fetal risks and risk exposures and the relationship to ASD risk.

Main effect variables of prior maternal smoke exposure and lactation were significantly associated with ASD, but results did not inform the concept of fetal programming or contribute evidence of plausible biological assault to support multi-threshold liability mechanisms. However, the association between prior maternal smoking, dedicated lactation and ASD was strengthened when adjusted when infant respiratory distress at birth. This optimized associative relationship to ASD risk was not significantly modified by inclusion of maternal age, preconception health status (i.e. maternal anemia, diabetes, high blood pressure, Vitamin B12 deficiency, neural tube defect risk, and albuminuria), and unique delivery risks such as preeclampsia or jaundice birth. Postdelivery infant traits such as infant sleep and breathing patterns did not confound the relationship of prior maternal smoking and lactation to ASD. Therefore, AGRE results informed temporality, but not causality of exposures shown in Figure 2.

In this study, risk exposures were abstracted, coded and analyzed from standardized data collection records for an adequate sample size, with adherence to all stakeholder internal review board (IRB) policies. Exposure parameters were compared within several independent archived datasets available for familial and non-familial controls and data were stratified by gender.

Research was needed to study the exposure-timing relationships of pregnancy factors, and ASD outcome, and the separate and combined effect modification of preconception, and neonatal factors among ASD cases and controls in an AGRE cohort. The relationship of pregnancy factors of maternal smoke exposure, maternal fish and multivitamin use, and dedicated lactation practice was studied with and without gender

stratification. Neonatal infant breathing traits were analyzed separately and in combination to determine the potential confounding effect on the relationship of pregnancy risk factors and ASD. Covariation and effect modification of preconception parental age, preexisting maternal health risks, and obstetric complications, and infant breathing on the relationship of pregnancy risk factors and ASD and the ASD gender differential was studied. A descriptive and bivariate analysis of these relationships may partially inform biologically plausible mechanism(s) of ASD etiology.

The details of the sampling strategy, statistical considerations, adequacy of sample size, and retrospective data abstraction, coding, and operationalization of survey items necessary to address the dissertation research questions will be described in Chapter 3. The hypothesized main effect relationship of pregnancy-related exposures to ASD outcome and the ASD differential was the initial data analysis focus. Study results contributed to the limited body of research related to the proposed effect relationship of maternal smoke exposure and diet during pregnancy, and lactation to ASD and the ASD gender differential. The hypothesized rationale for directionality of the relationship, impact of hypothesized confounding and covariate effects was described. Bivariate cross-tabulations, odd ratio analysis, and variable recoding and manipulation to support as logistical regression for ASD outcome were discussed in Chapter 3.

Chapter 3: Research Method

Introduction

There is replicated, suggestive evidence of differences in nervous system development, inflammation, and cytoskeletal organization among children with ASD compared to typically developing children and siblings (Abrahams & Geschwind, 2009; Gabory et al., 2009, Hu et al., 2009). However, the association between early life exposure proxy variables for these genetic, cellular, or metabolic processes, ASD, and gender within a well-characterized genetic cohort is lacking. The purpose of the study was to describe the exposure-timing relationship between pregnancy traits (maternal smoke exposure and diet during pregnancy and lactation) to ASD outcome, as mediated by genetically susceptible preconception parental age, maternal health, and obstetric complications, and as confounded by neonatal traits to the risk of ASD and the ASD gender differential. I aimed to contribute to the limited body of research on the hierarchical relationship of temporal factors, shared familial environmental risks, unique exposures and risk factors, and infant gender to the risk of ASD. Factors during pregnancy were assumed to be the main effect risk factors affecting ASD. Study questions and hypotheses presumed preconception factors were covariates or mediator variables, and neonatal traits confounded the relationship of independent or combined pregnancy factors to subsequent ASD.

The variables related to maternal diet during pregnancy were categorized by fish type and duration and frequency, and intrapregnancy multivitamin use. Preconception maternal health variables included self-reported responses to medical history questions

related to maternal iron deficiency, vitamin B12 deficiency, neural tube/alpha fetal protein/triple screen (AGRE's Medical History Survey, Section A, Question 55) and the temporal, binary response to medical questions related to maternal high blood pressure, anemia, diabetes, and albuminuria status (AGRE Medical History Section A, Questions 37, 48, 49 and 58, respectively).

The dependent or outcome variable was confirmed autism diagnosis as defined by *DSM-IV* or *ICD-9* criteria. Categorical treatment of the dependent variable included the validated AGRE classification of broad ASD, ADIR positive and *OR* ADOS positive criteria used as a dichotomous outcome. Only ADIR criteria were used since ADOS score data were incomplete.

In this chapter, I describe the case-control research design and rationale for the conceptual framework used to describe the hypothesis of the main effect relationships of pregnancy risk factors (maternal smoke exposure, diet, and lactation) to ASD outcome. Subsequently, the effect modifications of distal correlates and confounding variables were analyzed both independently and in combination of the relationship of pregnancy factors to ASD; then, the ASD gender differential was explored. Methodology for the use of archived datasets from the AGRE standardized survey instrument, with appropriate permissions, and the operationalization of survey items and coding of variables were also detailed. The a priori proposed use of ordinal indexes for variables to support multivariable regression is described. I conclude the chapter with a discussion of threats to study validity and ethical procedures necessary to implement the study in compliance with all related ethical, confidentiality, and stakeholder internal review board guidelines.

The proposed framework for the study was presented in Figure 2. Methodology, research design, and rationale for testing the hypotheses that three proxy variables of placental transfer mechanisms (maternal diet, smoke exposure before and during pregnancy, and lactation) were main effect variables in the relationship to ASD and gender differential of ASD and are described in detail. Statistical design and a priori treatment to address the confounding effect of neonatal traits on the pregnancy exposure-ASD relationship are addressed. Thirdly, the proposed effect and statistical treatment of the covariate, distal effect of preconception health factors such as parental age, prepregnancy maternal health (high blood pressure, diabetes, anemia, vitamin B12 deficiency, neural tube defect risk, and albuminuria) and two obstetric complications hypothesized to be genetically predetermined, on the relationship of maternal diet and smoke exposure during pregnancy and lactation to ASD were addressed.

The goal of the proposed retrospective, case-control design was to articulate an exposure-timing relationship for ASD, with minimal uncertainty bias, measurement, and recall bias with regard to health behaviors such as dietary intake, maternal household smoke exposure, and health status and adjustment for unique obstetric risks that may confound the relationship of dominant risk factors of ASD and the ASD gender differential. A second design goal was to achieve adequate statistical power and effect size to study the gender differential of ASD within a cohort representing a well-characterized genomic sampling frame.

Research Design and Rationale

The study design was a case-control design using retrospective datasets and pairs. Case-control design was appropriate for hypothesis testing, particularly among rare health outcomes. Case-control is a cost-effective design when health outcome etiology is unknown (Friis & Sellers, 2005). The design leveraged existing, archival, primary data sets that contained familial controls, well-matched nonfamilial controls, and repeated measures of temporal exposures. Although case-control designs often have the disadvantage of uncertainty of the exposure-outcome relationship, the hierarchical framework proposed to cluster temporal uncertainty of multiple exposures. Exposure-timing relationships were the basis of the hypothesized framework and were analyzed using proportional odds ratio analysis.

The source of cases and control groups was predetermined based on previous, independent AGRE researchers' study protocols. However, the study design was subject to risks of misclassification due to familial and nonfamilial sources of cases and controls (Friis & Sellers, 2005). The source of cases reflected possible recruitment and geographic bias as well as potential cohort effect related to access to medical treatment resources. However, as discussed in Chapter 2, the results of numerous AGRE studies were consistent with the risk factors and relative risk or prevalence rates identified in several cross-sectional studies within other U.S. and global cohort samples.

This study case-control study designs for bivariate analysis and multiple regression analysis for case selection criteria of broad binary ASD outcome (ADIR scores of 1 or 0). The design of preconception, pregnancy exposures, and early life stage

leveraged existing etiological data and explored hierarchical relationships and the gender differential. Internal parental response consistency validity was attempted by using antecedent and redundant responses to independent main effect variables. Logistical regression analysis of coded, ordinal values of independent variables was proposed and conducted after converting all variables to binary responses. Study results were reported as odds ratios with 95% (one tail) confidence intervals.

Case-control design with the broad ASD outcome (i.e., ADIR diagnosis only) was used in bivariate analysis. The use of archived retrospective data was expected to provide time and resource efficiency benefits, and the use of a familial registry minimized the constraint due to rarity of ASD sampling population accessibility. However, the reference group consisted of both nonfamilial and familial controls. Therefore, descriptive statistics for the diverse pooled control group were explored, as was additional posthoc statistical analysis.

It is common practice to study the relationship of broad versus strict ASD diagnosis in autism etiology studies using cross-sectional study designs. (Amin et al., 2011; Hallmayer et al., 2011; Kalkbrenner et al., 2012; Schultz et al., 2006; Wallace et al., 2008). But the ideal situation is to focus on a more narrow or restrictive case selection criteria in a case-control design (Friis & Sellers, 2005). In this study, the primary goal was to use the strict criteria and consistent exclusion criteria a priori. However, ADOS criteria for the ASD definition were not widely available for the AGRE data sets. Exposure proportions, tests of association, test of trends, were used to describe the relationship of main, covariate and confounding variables to binary ADIR outcome.

The proposed experimental design was a classic design used for rare disorders with less than five to ten percent population prevalence (Gerstman, 2008). The retrospective study design, with a binary outcome variable is a common design used to study ASD risk factors. The ability to cluster variables by temporal aspects of risk exposure, assumption of directionality of exposure risks, and the use of an archival registry chosen to provide adequate sample size to study the gender differential of ASD were other benefits for the choice of a case-control design.

An adequately powered case-control design was with the objective of providing adequate statistical sensitivity to detect gender-differential risks of preconception, main effect or confounding infant traits associated with ASD among females in the AGRE population. Gender differential of ASD has not been well-characterized due to inadequate statistical power of most previous studies identified in the literature (Adams et al., 2011; Evans et al., 2008; Hertz-Picciotto et. al., 2011).

In this dissertation, it was assumed that a focus on the interrelated exposure-timing of preconception, prenatal and early life exposures minimized biases published in many previous studies which attempted to identify potential biomarker variables. Previous biomarker studies associated with ASD often did not consider the temporal aspects of exposures, exposure or dietary dosage or duration, nutrient uptake, or dietary interactions (Ratjczak, 2011; Zerbo et al., 2013). The relationship of lactation to ASD and the ASD gender differential remains unclear.

Few previous cross-sectional or case-control studies on maternal smoking, maternal health status, or lactation duration adjusted for pre- or postnatal nutritional

fortification. This study proposed to investigate reported maternal intrapregnancy multivitamin use and weekly fish intake. However the availability of maternal fish intake data was a study constraint.

Another constraint of the case-control experimental design is the inability to address timing of exposures (Colditz, 2010; McDonald & Paul, 2010). Randomized controlled trials, and meta-regression studies are unable to address the exposure-disease relationship (Colditz, 2010). The application of a hierarchical framework to characterize timing and proxy ASD risk variables was proposed to understand the possible impact of timing of exposures among a well-characterized population. The plausibility of placental transfer mechanisms was of interest and was considered in the construction of the study and the first three research questions. Data for antecedent behavioral responses was used to validate subsequent behavior. Also, the proposed design and statistical power calculations had estimated the sample size and effect size required to explicitly study the gender differential of ASD. One study objective was to explore biologically plausible rationale for the gender differential of autism by studying temporal factors, potential placental transfer risks, and possible gender-specific neonatal traits.

The comparability and confounding health status of control groups in several autism studies has been challenged and debated (Kocovska et al., 2012). In this study, the use of controls included nonaffected siblings, with similar shared household exposure variables and genetic inheritance even if not specifically age-matched. In addition, univariate analysis tested whether the AGRE sampling frame characteristics reflect typical U.S. prevalence rates of breastfeeding duration, multivitamin use, and smoke

tobacco use previously reported by U.S. demographic trends (D'Angelo et al., 2007; Kalkbrenner et al., 2012; Tong et al., 2011; Wiest, 2007). Several researchers have compared the AGRE population demographics to other autism sampling frames (Durkin et al., 2008) and reported no systematic biases (Cantor et al., 2007; Wallace et al., 2008).

The use of data for unaffected siblings as controls may have mediated uncertainty and misclassification bias for variables associated with shared environment factors. The use of three AGRE data sources for prior smoking behavior minimized recall bias. However, no assumptions were made with regard to birth-specific smoking or lactation behavior. Most previous ASD etiology studies did not directly address the ASD gender differential as an effect modifier of other risk variables. Misclassification bias was minimized by the use of familial controls.

Internal validity and risk of misclassification bias was addressed by the presumed framework of main effect, distal covariates, and potential confounding factors, which represented potentially overlapping maternal and fetal risk factors. However, it was hypothesized the temporal aspect of these factors varied, such that the variables do not likely represent repeated measures of the underlying risk factors. It was presumed in the AGRE study design that obstetric complications were predetermined risks that likely were associated with preconception maternal health status.

The use of large, representative AGRE datasets and the use of ADIR outcome and "Unaffected Sib" records were used in attempt to reduce threats to internal validity. Data pooling of substudies among independent researchers of the AGRE registry was not available but was proposed to minimize matching biases, sampling, uncertainty, and

misclassification bias. Overall record count suggested an adequate sample size to study ASD gender-differentials; but many control records had missing main variable values.

Methodology

Study Population

Autism Speaks' AGRE is an open source, nonprofit DNA repository and family registry database of genotypic and phenotypic information that is available to autism researchers worldwide (Lajonchere, 2012). The research consortium program began in 1997 for families who have two or more children on the autism spectrum (Lajonchere, 2010). Referrals from clinical and medical professionals are the primary ascertainment method and there is no restriction to age, ethnicity, or socioeconomic status. As long as there are two affected family members, parity is not considered. Over 2,000 families have participated in the program representing the 50 United States. As of 2007, the majority of the sample (75%) was Caucasian and nonHispanic, and 37% of families were from the West Coast (Cantor et al., 2007; Lajonchere 2010). Autism affected offspring in the AGRE were all born since 1992 (Cantor et al., 2007; Geschwind et al., 2001; Stone et al., 2004). ASD criteria are limited to *DSM-IV TR*, ICD-9 codes for strict and broad ASD.

As an approved, independent research applicant to the open access, AGRE research community, I obtained access to parental self-reported family records acquired through clinical care for recruited registry participants. AGRE registry datasets also contained information on sibling and nonsibling matched controls for children diagnosed with autism disorder. The complete registry contains over 3,000 families within the United States characterized by having at least one English-speaking parent (Lajonchere,

2012). I had access to 902 affected child records. Separate data files were obtained for mother's history, father's history, and childhood records.

The open access autism registry based on AGRE and National Institute of Mental Health (NIMH) repositories was implemented with standardized autism diagnostic criteria with minimal sampling bias and has been extensively researched for genetic traits but with minimal (i.e., less than 10% of studies) performing gender-stratified analysis (Lajohnchere, 2010). Medical records reflect prospective and retrospective clinical data by physician interview whereas parental self-report surveys reflect retrospective behavioral data. During enrollment to the AGRE program, parents completed multiple self-reported questionnaires regarding dietary and social behaviors. These records were supplemented with medical records from referral and supplemental physician visits (Lajonchere, 2010). While the primary purpose of the AGRE registry has been to identify genome-wide association tests based on genetic and allele profiles, there is merit in exploring the extensive archived medical records for tests of associations based on the infant life cycle timing of exposures. Measures reported for antecedent exposure periods may improve data validity.

Sampling Strategy

Proposed sampling procedures were based on accessibility to databases used by previous autonomous research group sub data sets with the AGRE registry. The proposed a priori multistage clustering using the AGRE sampling frames included those of Carayol et al. (2011), Stone et al. (2004 and 2007), and Yonan et al. (2003) was not feasible. Rather, access to several raw data files were used as described in Chapter 4, to

obtain an aggregated raw data set of records, and values for the proposed nine variables for AGRE cases and controls. Parental reported data for the abstracted variables were collected, merged, and cross-verified by parental history for cases and controls by ADIR status. Participant inclusion criterion was established a priori as families with at least one English speaking parent, with childhood cases born since 1992. Exclusion criteria systematically defined by prior researchers and the AGRE Steering Committee (Geschwind et al., 2001) were used in this study. Typical exclusion criteria included as single-gene disorders, regressive ASD, and specific congenital gene mutations such as Rett's, Prader-Willis, Angelman's syndrome, Timothy Syndrome, Fragile-X, phenylketonuria and tuberous sclerosis; and exclusion of subjects with Wescler's Intelligence Quotient scores less than 70 (Carayol et al., 2011; Stone et al., 2004 and 2007; Yonan et al., 2003).

Sampling Power Analysis

The effect size for many behavioral and social science variables is a study challenge (Lipsey & Wilson, 1993). In this study, the effect size of key variables of interest was summarized and described in Table 3. The results in Table 3 suggested the relationship between maternal diet, and in particular multivitamin use and ASD diagnosis in offspring was expected to be the most difficult effect to identify in this proposed study. Few studies described in Table 3 used continuous values for key variables which further complicated calculations and estimates of adequate sample size for optimal statistical power. The study presumed a standard Type I error value of $p = 0.05$, with evidence based justification for one-tailed hypotheses assumptions. Therefore, directional one-

tailed hypotheses were rationalized for the nine proposed variables. Risks of maternal smoking and, low fish and vitamin intake during pregnancy, adjusted for increased maternal age, smoking history, obstetric complications, and infant male gender, were suspected ASD risk factors. Lactation was expected to be a preventive measure against ASD diagnosis. It was hypothesized gender specific risk factors may include obstetric complications.

The effect size between cases and controls, and the effect size for the gender-differential were estimated using approximated Cohen's *d values* (group mean differences divided by averaged standard error) for differences in fatty acids among cases and controls, and among females and males. Estimated Cohen's *d values* were calculated for data in studies reporting quantitative group-differences in plasma levels of fatty acids in ASD cases and controls (Wiest, 2007; Wiest et al., 2009), plasma sphingomyelins in males and females as indicated in Table 2 (Mittlestrass et al., 2011), and dietary estimation of fatty acid intake in pregnant and nonpregnant women, and adults and children adjusted for protein intake (Novak & Innis, 2012).

Cohen's *d value* estimates the gender differences in plasma DHA and arachidonic acid levels was roughly 2.0 to 4.0 among cases and controls (Weist et al., 2009). Weist (2007) suggested plasma EPA levels were elevated in 2 of 12 (16.7%) of cases which was likely associated with the self-reported use of krill or fish oil dietary supplements. However, a normalized distribution of plasma EPA levels was reported for 15 of 238 subjects who reportedly consumed fish at least once per week (Wiest, 2007). Cohen's *d value* estimates for quantified levels, based on dietary recall of particular fatty acids

(DHA, and EPA) among pregnant and nonpregnant women, and children and adults in a cross-sectional study of the association of dietary fatty acids, protein and fat-energy intake were also estimated (Novak & Innis, 2012). Cohen's *d value* estimates of 0.2 were calculated for dietary estimated levels of DPA and EPA levels among pregnant and non-pregnant women; whereas the Cohen's *d value* calculation by gender was much higher (0.7 and 1.3 for EPA and DHA, respectively). Given the assumption of a one-tailed test, and *t-test* for related samples as reflective of an assumption that sibling controls may be the predominant matching criteria in the AGRE cohort studies, the sample size was approximated from standard tables. For the assumption of a Cohen's *d value* of 0.2, and $1-\beta = 0.90$, an estimated sample size of 133 cases and controls for related or matched samples. If the assumption were re-estimated using a Cohen's *d value* of 0.3, the estimated sample size for adequate power would be 60 each for cases and controls for a one-tailed test.

The sample size needed for minimum detectable difference estimates between cases and controls, or between genders of children with ASD were estimated by the equation below (Gerstman 2008, p 252):

$$\Delta = \sqrt{[2 * \sigma^2]/n * (Z_{1-\beta} + Z_{1-\alpha})} \text{ solving for "n" per treatment}$$

when $\alpha = 0.05$ (one-sided) and

power = 0.80 and assuming independent group means.

Assuming a mean difference of 0.2, standard deviation of three units (0.6) based on the Chebychev's rule of normality and standard deviation variance (Gerstman 2008, p 81):

$$N = 2(0.6)^2 * (0.76 + 1.96)^2 / 0.2^2 = 133 \text{ each for cases and controls; or by gender.}$$

An alternative method of estimating the total sample size needed for 80% statistical power was estimated by the number of variables in the proposed study (Tabachnick & Fidell, 2000). A fatty acid marker, levels of plasma sphingomyelin, was measured by gender in healthy adults (Mittlestrass et al., 2011). Gender-differentiated plasma sphingomyelin levels differed among healthy adult males and females with a correlation of 0.28 to 0.39 (Mittlestrass et al., 2011). Therefore, the estimated total number of subjects, given the number of proposed variables (nine) and conservatively assuming a correlation coefficient (R^2 of 0.13) was calculated as follows (Tabachnick & Fidell, 2000): $N > (8/f^2) + (m+1)$ where $f^2 = R^2/(1-R^2)$ and where

m = number of variables and let $R^2 = 0.13$ such that $f = 0.02232$

therefore $N > 358 + (9-1)$ or $N > 366$ subjects total

Thus, the ideal sample size required to provide 80% power and control Type 1 error rates ($p = 0.025$) was expected to be at least 60 to 133 girls, among a sample cohort size of at least 120 to 265 subjects. A targeted ideal subsample size at least 265 female children, after exclusion criteria and data cleaning for missing variables was an initial study design objective. Ideally, a final sample size of 175 girls and 400 children, may have accounted for a 20-30% rate of exclusion due to comorbidities or IQ scores less than 70, or missing values, to reflect a final goal of 80% power, 0.5 effect level and alpha (type I error rate) of 0.025 statistical test. The final sample size ($n=733$ records) reflected 556 male children and 177 female offspring. Parental self-report data was not matched with physician collected AGRE data to minimize missing data. The target sample size

necessitated the use of pooled archival AGRE cohort populations used previously (Cantor et al., 2007; Carayol et al., 2011; Hallmayer et al., 2011).

Archival Data Sources

AGRE subjects and parents completed extensive self-report surveys on family, parental and child behavior, diet, and exposures which may be complimented with medical records from referral physicians (Lajonchere, 2010). Parental self-reported data system was collected in the Online System for Clinical Research (OSCR). Standardized evaluation OSCR survey tools existed for “AGRE Lifestyle” survey questions which included maternal and household smoking behavior, prepregnancy health status (See Appendix B), “AGRE Medical History- Child” which included maternal prenatal birth control and reproductive practice questions, pre-and postnatal vitamin supplementation, and neonatal delivery questions such as preeclampsia and jaundice birth (See Appendix C). OSCR standardized abstracted evaluation protocol for ”AGRE Metals and Mother’s Diet” was limited to questions about frequency, duration and types of fish. Data sets from five AGRE survey tools (see Figure 3) were used to extract variable values proposed in this study. The average age of children with ADIR and ADOS diagnosis within the AGRE cohort was expected to be 6 to 8.68 years, for data representing a sample of 444 children with a group gender ratio of 3.5:1 males to female (Wallace et al., 2008). Using ADIR criteria, the average age of subjects ranged from 9.1 to 10.2 years in this study.

Operationalized AGRE Survey Instrument

Parents, with physician referral, were administered self-reported OSCR surveys to be completed retrospectively upon enrollment to AGRE (Cantor et al., 2007). Table 4

describes proposed independent variables, archival AGRE survey items, scales and coding to be extracted and defined as exposure-timing variables for preconception, pregnancy, and infant traits in this proposal. The first four variables listed vertically in the first column reflect pregnancy exposures to be individually analyzed in the relationship to ASD and the ASD gender differential. The next three variables in the first column (preconception parental age, preconception maternal health, and obstetric health) were tested, separately and in combination for the covariation effect on the relationship of pregnancy factors to ASD outcome. Confounding effect of infant sleeping and breathing pattern, on the relationship of pregnancy factors to ASD were analyzed using the last two variables listed in the first column of Table 4.

Wording of the specific surveys and questions identified a priori in the third column of Table 4 are illustrated in Appendices A, B, C, D, and E. Similar AGRE survey behavioral or medical questions were often asked with reference to preconception, pregnancy, and neonatal periods. The responses for antecedent periods for a given question was expected to be used to verify internal validity of subsequent responses and to minimize missing variable values. For example, parental smoking for a multiple birth pregnancy was considered similar among all births in that pregnancy. Maternal and paternal self-reported smoking behavior were compared for "prior" or "ever" smoke periods. The degree lactation dedication (i.e., no bottle use) in the AGRE survey was used to validate responses to lactation duration. Supplemental specific AGRE questions related to artificial reproductive technology use were used to further study the relationship of parental age and obstetric risk and ASD risk. The study assumed obstetric

complications were predetermined, directly through preconception factors associated with genetic susceptibility rather than neonatal traits. Actual data available varied somewhat from the variables listed in Table 4. Any discrepancies or differences from the a priori variable definition and final data definitions are explained in greater detail in Chapter 4.

Table 4

Study Variables, AGRE Survey Items, and Proposed Variable Definitions

Independent variable	Operationalized AGRE value scale	AGRE survey and item/question (q) ID	Proposed coding and possible indexing values
Maternal Smoke Exposure During Three Trimesters	Nonsmoking (ever) Cigarettes per day by pregnancy trimester	Lifestyle Sec. A q 1-18: and Medical History-Child Sec. B, q 6/7	Maternal, paternal, household smoking (never, prior, ≤ 10 , > 10 cigs/day) during pregnancy trimesters (4 points maximum/parent)
Maternal Diet During Pregnancy And Lactation	Fish intake \leq once/wk Fish intake $>$ once/wk for all 17 types of fish Prenatal vitamins use (yes/no) by trimester	Metals and Mother Sec A, q 1-8 Sec A., q 122/123 Med. Hist Child, Sec. A q 121-124, Sec. B q 10-12	Maternal diet (0 - 4 or 2x2 pts) (two points for "yes" on each of fish & vitamin factors present in last trimester)
Lactation	Unknown None Less than two months Two to six months More than six months	Medical History-Child Sec. B q 1-7 Sec. B q 16-23	Lactation Index (0 -7 points) Lactation Duration (0, 1, 2, 3, 4) score plus Non/Dedicated, Bottle Use (DK, Non/dedicated): 3 points
Infant Gender	Male or Female	Child Demographic Survey	Gender (2 points for male)
Parental Age at First Child Birth	Father: 20-29,30-39, 40-49 : < 20 or > 49 Mother: < 36 years at singleton first child birth	Medical History of Father Medical History of Mother/Child Demographic Survey Sec. B q 8-18 (ART use)	Paternal age (< 20 , 20-48, > 48) at first birth (3 points max) Maternal age (≤ 36 , > 36 yrs) at first birth (2 points max)
Preconception Maternal Health Before Pregnancy	High blood pressure Diabetes Low iron/Anemia Vitamin B12 deficiency Neural tube defect risk Diagnosed albuminuria	Med. History-Mom Sec. A, q 37,q 49, q 55 Med. History-Child Sec. A, q 23/24, 48/49 Sec. A. q 58/59	Preconception Index (0-10 pts) (two points for "yes" response on each of 5 diagnosed factors present before affected pregnancy)
Obstetric Health	Preeclampsia Jaundice delivery	Med. Hist.Child Sec. A, q 90, 166. Med. Hist. Child Sec. A, q 90-92	Obstetric Index (0 - 4 points) (two points for "yes" on each factors present $>$ third trimester)
Infant Sleep Pattern	Regular? Yes/No Onset/diagnosis (0-11 mo, 1- 8, > 8 yrs) Snoring- Yes/No, age	Medical History-Child Sec. B q 31-39, Sec. C q 31, 31-39, Sec. C, q 80	IrregSleep/Snore (0 -4 points) (0,1,2): 2 points for each factor If present at < 11 months age
Infant Breathing Pattern	Regular?- Yes/No Shortness of Breath- Yes/No, age	Medical History-Child Sec. B q 31-39, Sec. C, q 32-39	IrregBreathing (0 - 4 points) (0, 1,2): 2 points for each factor if present at < 11 months age

Note. Sec= Section; cigs= cigarettes; ART= artificial reproductive technology; DK= don't know.

For all univariate, bivariate and regression analysis, the dependent, dichotomous outcome variable was confirmed autism diagnosis as defined by *DSM-IV* or *ICD-9* criteria. Binary outcome responses for “broad” ASD case ascertainment (ADIR score) was used for data analysis. Binary categorical treatment of the dependent variable include the validated AGRE classification of broad ASD; “ADIR positive” criteria (American Psychiatric Association, 1994). Case ASD outcome exclusion criteria included child or parental Fragile X, trisomy or quadrupled 15q11-13, trisomy 21 & Xp22.3, phenylketonuria (PKU), Rett or Tourette's syndrome, Tuberous sclerosis, Angelman's, Timothy Syndrome, Prader-Willis, mental retardation and Wechsler Intelligence Scale for Children score less than 70 as recorded on AGRE Child Medical History Survey, Section C, Question number 93 (See Appendix C).

Within the original, available AGRE survey tool datasets, survey responses for variables were scaled and recorded as dichotomous (yes/no) responses, categorical duration (1-3 months before pregnancy, first, second, third trimester, and during lactation, number of cigarettes or smokes per day, dietary frequency intake per week and servings per day), or continuous variables (age, weight, time/duration and degree of lactation dedication, temporality and daily frequency of smoking, and/or dietary practices). Responses for “blanks” were primarily cross-checked with other survey items by child record. Responses for the previous temporal period were used to assess internal data validity of exposure-frequency for the particular period of interest. AGRE data was available for antecedent values of smoke exposure before or during pregnancy, maternal diet before or between pregnancies and neonatal traits by child age. Neonatal infant

sleeping or breathing responses for a given children for the period of 1 to 8 years was compared to infant sleeping and breathing responses for the same child for the period at 0 to 11 months. However, only neonatal breathing traits at birth or delivery were used in statistical analysis. Categorical treatment for parental and/or maternal smoking was replicated from previous recent studies on ADHD outcomes for AGRE and nonAGRE cohorts which used the typical categories of nonsmoking, mean of 10, or mean of 20 cigarettes/day (D'Angelo et al., 2007; Habek & Kovacevic, 2011; Kalkbrenner et al., 2012; Lindblad & Hjern, 2010). There was multimodality of the distribution of cigarette consumption variable within the AGRE datasets. As a contingency protocol, it was suggested a priori that ordinal recoding be defined as "never, prior, < 10 cigarettes/day". Due to data multimodality, the data range for prior maternal smoke exposure variable, the highest ordinal category was defined as "greater than 10 cigarettes/day". It was anticipated the maternal smoke exposure variable would be (as it was) collapsed to ordinal and/or dichotomous response to conduct multivariable regression analysis.

Categorical treatment of maternal fish intake was expected to reflect the total intake of 17 fish types (mackerel, tilefish, swordfish, shark, marlin, tuna, bass, catfish, cod, crab, lobster, salmon, trout, mahi mahi, pollock, other freshwater or farm-raised fish) described in the AGRE survey instrument. Total fish intake frequency was to be summed over three trimesters to define the fish intake during pregnancy variable. Antecedent values of fish intake and frequency for one to three months prior to affected conception was planned to be used to assess internal validity of fish intake during pregnancy and breastfeeding. Responses for fish intake during lactation were also be used to test internal

validity of lactation duration and exclusivity variables. However, availability of maternal fish intake data for subjects in this study was very limited. Therefore, the analysis of maternal fish intake was not included in bivariate results of maternal diet factors.

Maternal multivitamin use was a dichotomous variable in this study and reflected the response for multivitamin use before, during pregnancy, and intrapregnancy periods. Dichotomous response to the question of maternal use of Vitamins D and E was not made available from the AGRE Metals and Mother Survey, Section B for preconception, pregnancy, and lactation periods. Fish oil supplementation was not described as specific form of dietary supplement in the survey, but was volunteered as a descriptive response for the category of “other”. Vitamin D, E, and fish oil data measured during preconception period was not made available for validation of maternal multivitamin use during pregnancy and neonatal period.

The categorical treatment for lactation duration was documented within AGRE OSCR datasets and was expected to include the continuous variables of infant age of initiation and termination of lactation, smoking during lactation, vitamin and supplementation during lactation (See Appendix C, Section B, Questions 1-16) and degree dedication to lactation, as well as infant formula brand. Lactation practice did not address other fluid intake beyond "bottle-fed", or "breastfed" practice options. Responses to lactation duration were internally cross-verified using related questions about lactation practice and exclusivity. Very few studies have focused on the relationships of lactation duration and dedication, to ASD, or the gender differential of ASD. The AGRE lactation duration categories were collapsed to reproduce the five-category protocol of Schultz et

al. (2006) shown in Table 3; “unknown, none, less than 2 months, 2-6 months, more than 6 months” who reported an inverse odds ratio relationship to ASD.

After initial descriptive and bivariate statistical analysis of parental age data, the variable parental age at first birth was coded in ways similar to that conducted by previous AGRE researchers (Anello et al., 2009; Cantor et al., 2005; Cantor et al., 2007; Wallace et al., 2008). For independent variables not previously identified or fully described in published AGRE literature, categorical treatment described in sentinel articles identified in this literature review was replicated in this study. All variable manipulation protocols were proposed and described below; in keeping with a priori procedures and literature precedence. The use of parental age categorical ranges is a typical data manipulation approach. In this study, categorical treatment of the AGRE variable parental age was replicated as ordinal values as described by Cantor et al. (2007) to defined ordinal ranks including; paternal age 20-29, 30-39, 40-49 years and mothers age as less than or greater than 36 years at singleton first child birth. Parental age groups were later collapsed to binary values using age cutoff of 36 years.

Preconception maternal health before pregnancy was proposed to be defined as the total of dichotomous values for each mother, for absence or presence of high blood pressure, diabetes, low iron, vitamin B12 deficiency, neural tube defect, diagnosed anemia or albuminuria for the period one to three months before affected pregnancy. Wallace et al. (2008) reported anemia variables were not missing in more than 5% of subjects, but anemia prevalence was too infrequent to be included in mixed effect models for an AGRE cohort of 444 affected offspring. In two other California cohorts,

preconception hypertension prevalence ranged from 3.5 to 3.7% and maternal diabetes prevalence range was 9.3 to 11.6% for moms with affected offspring born from 2003 to 2010 (Krakowiak et al., 2012). Lawrence et al. (2008) reported preexisting diabetes prevalence was 1.3% and gestational diabetes prevalence was 7.6% for a southern California population cohort of pregnant women for the period 1999 to 2005. Dodds et al. (2011) reported preconception diabetes was 0.4 -0.8% for mothers of controls and cases respectively; but gestational diabetes was 2.7 and 3.5% for mothers of controls and ASD cases in the Canadian cohort of infants born between 1990 and 2002. It was therefore presumed in this study that comorbidity of conditions during the preconception period would adjust for unobserved but possible covariate, gestational diabetes.

AGRE categorical treatment of obstetric complications of preeclampsia, jaundice, and albuminuria, was replicated as described by Wallace et al. (2008). The responses for eclampsia and preeclampsia were combined across trimesters to reflect a single variable. The AGRE researchers reported infrequent responses on Apgar score, resuscitation, and mechanical ventilation at delivery and more than five percent missing values for pregnancy infections. The researchers suggested an optimality index for obstetric factors was a viable approach used by other researchers. But it was reportedly not feasible in their study of 19 obstetric risk factors since an additional 16 obstetric health variables had high missing values.

The characterization of infant sleeping and breathing patterns were incorporated as potential confounders that may reflect birth-specific comorbid conditions such as asthma, congenital heart defects, pharmacotherapy use, allergies, or comorbid mental

disorders (Liu, Hubbard, Fabes, & Adam, 2006). Few survey instruments or observational measures have been standardized to address sleep and breathing disorders; particularly among infants (Mahoney & Caterino 2011; Young et al., 2009). Response variables for infant breathing and sleep problems were collected in the AGRE Child Medical History survey (see Appendix C, Section B, Questions 32-39) as binary responses (irregular or regular sleep pattern) with a supplemental dichotomous question about snoring, and regular or irregular breathing, with a supplemental binary question about shortness of breath (for age in months and years). Categorical responses for infant sleep pattern were expected to be recoded and validate previous research related to risk of sudden infant death and hypoxia (Carolan & Bye, 2011); “0-2 months, 2-4 months, 4-9 months, 9-12 months, > 12 months”. Due to missing and incomplete data, this AGRE study used dichotomous responses for infant respiratory distress at birth, and resuscitation required at delivery. It was shown that sleeping and breathing patterns for infants (0 to 11 months), validated by responses for subsequent age periods for the specific participant.

Manipulation of Operationalized Data

The framework proposed in Figure 2 was constructed to study the exposure-timing relationships of pregnancy, preconception, and neonatal factors, both separately and in combination among ASD cases and controls; with fetal or infant gender stratification. Therefore, after initial bivariate analysis to determine the interaction, covariate and confounding relationships, aggregate indices of exposure-timing variables (preconception, pregnancy, and neonatal factors) were analyzed. Prior to collapsing

smoking frequency (cigarettes per day) and lactation duration (months) to ordinal values, frequency distributions were inspected.

Supplemental ISAAC information was not available, and the frequency of missing values reduced statistical power, so each of the independent variables were recoded, as one-way, directional ordinal values as illustrated in the last column in Table 4. After recoding, responses for “don’t know”, blank responses, or “not applicable” were excluded which resulted in 733 completed records extracted from 902 raw patient records. Independent variables were coded such that higher index score values reflected hypothesized adverse or higher ASD risk. An aggregate risk index score for each of the covariate and confounding exposure-timing categories (preconception, and neonatal period) was initially proposed, but not needed.

Given the proposed coding scheme in the last column of Table 4, which was created as a contingency plan in the event of inadequate statistical power, risk factors were initially proposed to be summed into one cumulative score. However, adequate statistical power was achieved in the study, and therefore, with the exception of posthoc analysis of obstetric risks, data variables reflected binary values for all study variables, and ordinal categories for lactation and smoking durations. Overall obstetric ‘optimality’ as related to PPDs has been used to weight each risk factor equally to provide overall pregnancy risk (Stein, Weizman, Ring, & Barak, 2006; Zwaigenbaum et. al., 2002). Overall obstetric risk index may be useful when risk factors are highly intercorrelated and difficult to evaluate independently, but may mask underlying confounding or interaction factors (Dodds et al., 2011). Dodds et al. reported the use of an aggregated optimality

index of prenatal, obstetric, and neonatal factors did not affect the relationship of individual risk factors to ASD diagnosis in a case-control study of 924 children with ASD. Ordinal obstetric risk indices were proposed for use within AGRE cohorts as was previously reported for the AGRE sample frame (Wallace et al., 2008).

Raw data for preconception risk factors obtained from AGRE medical records did not impact the relationship of pregnancy and lactation factors to ASD, and missing values were minimal. Therefore this information was treated as indexed ordinal variables to improve statistical power in multivariable regression analysis. An indexed overall preconception proxy risk variable was a viable approach to reduce Type II statistical error, and minimize known confounding of obstetric complications associated with birth order, gestational age, birth weight, and Apgar score on ASD outcome in the AGRE cohort (Wallace et al., 2008).

For this dissertation, proposed composite scores were shown in the final column of Table 4 and included obstetric index scale of 0, 1, or 2 points to reflect preeclampsia and jaundice. After data coding, a proposed preconception health index of 0, 1, 2, 3, 4, or 5 points for the separate medical history questions related to maternal high blood pressure, diabetes, low iron/anemia, vitamin B12 deficiency, neural tube defect risk, diagnosed anemia, and diagnosed albuminuria was calculated as indicated in the last column of Table 4. Coded measures of obstetric and preconception risk covariates were analyzed as binary values, and as categorical values as explained in Chapter 4.

Data Management and Analysis

Burchinal and Neebe (2006) recommended NIH and FDA based data management guidelines that included using unique ID identifiers and a master file system that revalidated ID variables such as gender and birth date for each subfile and directory (Burchinal & Neebe, 2006). AGRE is supported by the NIH repository system design, NDAR, and is assumed to include separate file retention for programs, AGRE also participated in a project to create global unique identifiers to link ASD clinical collections (Johnson, et al., 2010). Appendices A, B, C, D, and E indicate the original codebook and data dictionaries for the archived AGRE instruments. Data source files were imported from AGRE's On-line System for Clinical Research (OSCR) using Microsoft Excel, a SPSS-compatible file form. Raw data, variable view, and data views within SPSS, statistical, syntax, and file documentation and print directories; with each updated version saved with a unique filename and date stamped for each thumb drive and hard drive directory per suggested protocol (Burchinal & Neebe, 2006).

SPSS version 21.0, under annual license of Walden University, was used. Syntax, code book, and recoding was documented using descriptive and date-stamped file names. A journal binder, chronically organized and listing all required data forms and variables, data check code and cleaning (range checking) data was used with consistent labels used for variables across all software programs, data sets, metadata documentation, and print files. Lab notebook tracking systems were used to follow progress in data collection to document which data were missing and why for a particular date or time. Data subfiles and program subroutines were described in a lab notebook to ensure there was clear

documentation regarding any data manipulation of electronic files, codebooks or annotated survey forms (Burchinal & Neebe, 2006). Assumptions and description of files, and cell or record counts for the treatment of "missing data" were documented and arranged chronologically in laboratory journal and electronic file systems.

Univariate descriptive analysis was used to check for data completeness, mean, median, standard deviations, outlier values, distributional assumptions, and external validity against broader U.S. population prevalence rates of smoking, lactation, maternal vitamin and supplement use, and obstetric complications. All data coding was dated and recorded in the journal.

After conducting univariate descriptive analysis, subset pooling and adjustment for missing values was needed for some variables. Treatment of missing values did not require the use of the Estimation-Maximization algorithm protocol published by Burstyn et al. (2011). Those researchers assumed variable values were missing at random, but conditioned on covariates and the outcome variable of ASD diagnosis.

Bayesian treatment of missing values for main effect variables may have been a more valid assumption (but not used), as evidence suggested reported maternal smoking behavior may have been "not-missing-at-random". Paternal self-reported smoking behavior tended to overreport smoking compared to maternal reports of "ever" or "prior" smoking behavior. In attempt to minimize small sample, model dependent estimates, an assumption was theorized a priori that mothers may fail to report smoking, inconsistent use of multivitamins, and lactation prevalence rates similar to those reported in U.S.

cohorts (D'Angelo et al., 2007; Dietz et al., 2011; Hauk et al., 2011; Kalbrenner et al., 2012; Lee et al., 2012; Tong et al., 2011; Wiest, 2007).

Bivariate descriptive analysis was conducted between main effect variables of maternal vitamin use, maternal prior smoking practice, exclusive lactation practice, and infant gender. Covariate and collinearity analysis, and collinearity tests were conducted for hypothesized covariates (maternal age, preconception health, obstetric complications) and covariates identified posthoc (i.e., gravida, multiple birth pregnancies, infant respiratory distress at birth).

The identification of mediator or distal correlate variables, which may affect the relationship of pregnancy and lactation exposures and ASD was also tested and controlled using the statistical technique of MacKinnon, Lockwood, Hoffman, West, and Sheets (2002). Statistical confounding and interaction was tested using the stratified bivariate analysis method (Gerstman, 2000). Statistical interactions were not identified in this study among independent variables. Thus, aggregate odds ratio analysis was appropriate. For prior smoke frequency (cigarettes per day), and exclusive lactation duration (months) strata-specific measures of association were reported for the bivariate analysis (Gerstman). An advantage of logit regression for data analysis was that exact probabilities are estimated, without the need for normal distribution of the diverse range of independent variables (Halpern & Visintainer, 2003). This was a study design advantage since several proposed independent variables for preconception health and obstetric complications were recoded as ordinal values or categories.

Research questions and hypotheses. The research questions addressed exposure-timing relationships of pregnancy and ASD outcome, and ASD gender differential, as adjusted for hypothesized confounding neonatal factors and adjusted for theorized covariates of preconception risk. Based on the independent variables of maternal smoke exposure and diet during pregnancy, lactation, and gender and their combination, subhypotheses were proposed. The specific study question and hypotheses were stated below for ASD outcome, with theorized confounders and covariates described in detail. Presumed and posthoc identified covariate preconception factors, and confounding infant traits were also be analyzed independently, and in combination to inform the relationship to ASD and ASD gender risk.

The initial research questions addressed: "What is the exposure-timing relationship between pregnancy factors, individually or in combination and ASD outcome within the AGRE sample cohort?" Secondly, it was of interest whether the relationship of pregnancy factors to ASD outcome is confounded by neonatal traits. The third series of questions tested whether preconception factors are effect modifiers of the relationship of pregnancy factors and ASD.

The initial three research questions addressed whether there is a statistically significant association between pregnancy factors such as maternal multivitamin use, prior maternal smoke exposure, exclusive lactation practice and the outcome variable, broad ASD diagnosis. These questions presumed a primary, main effect relationship of pregnancy factors to ASD risk.

Research Question 1: What is the relationship between maternal smoke exposure before or during pregnancy and ASD risk in offspring within the AGRE cohort?

H_{01} : There is no association between prior maternal smoke exposure and ASD.

H_{a1} : There is a positive association between prior maternal smoke exposure and offspring ASD in the AGRE cohort.

Variables: *Maternal smoke exposure (0, prior, ≤ 10 or > 10 cigarettes/day);*
Prior maternal smoke exposure
 ASD outcome: broad definition of ASD (ADIR score).

Research Question 2: What is the relationship between maternal multivitamin intake during or between pregnancy and ASD risk in offspring within the AGRE cohort?

H_{02} : There is no association between maternal multivitamin intake and ASD.

H_{a2} : There is an inverse association between maternal multivitamin intake and offspring ASD in the AGRE cohort.

Variables: *Maternal multivitamin intake (yes/no) during or between pregnancy,*
 ASD outcome: broad definition of ASD (ADIR score).

Research Question 3: What is the relationship between lactation and offspring ASD risk?

H_{03} : There is no association between lactation and offspring ASD risk.

H_{a3} : There is an inverse association between lactation and offspring ASD risk.

Variables: *Lactation duration (none, less than 2 months, 2-6 months, > 6 months),*
Lactation exclusivity (nondedicated, dedicated),
 ASD outcome: broad definition of ASD (ADIR score).

After exploring the hypothesized primary relationship of pregnancy related variables to ASD outcome, the most robust and significant relationship was carried forward for further study. The potential confounding of neonatal breathing traits and the effect

modification of preconception risk factors were tested in subsequent analysis, for the broad ASD outcome criteria (ADIR).

The fourth research question addressed whether neonatal sleeping or breathing traits confounded the relationship of pregnancy factors to offspring ASD outcome..

Research Question 4. How is the exposure-timing relationship of pregnancy variables (maternal smoke exposure, diet, and lactation) to ASD confounded by neonatal infant sleeping or breathing traits within the AGRE cohort when analyzed separately or in combination?

H₀4: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) and ASD risk is not confounded by infant sleeping or breathing traits.

H_a4: The relationship between pregnancy exposures (maternal smoke exposure, diet, and, lactation) and ASD risk is confounded by infant sleeping or breathing traits.

Variables: Prior maternal smoke exposure (yes/no),

Maternal multivitamin intake during or between pregnancy (yes/no),

Dedicated lactation (yes/no),

Infant respiratory distress at birth (yes/no)

ASD outcome: broad definition of ASD (ADIR score).

The fifth research question addressed whether infant gender mediated the effect of pregnancy related variables and ASD diagnosis among cases and controls in the cohort.

Research Question 5: How does the exposure-timing relationship of pregnancy variables (maternal smoke exposure, diet, and lactation) to ASD outcome differ by infant gender?

H₀5: The relationship of maternal diet, prior smoke exposure, and lactation to ASD outcome does not vary by infant gender.

H_a5: The relationship of maternal diet, prior smoke exposure, and lactation to ASD outcome does vary by infant gender (i.e., higher in males).

Variables: Prior maternal smoke exposure (yes/no),
 Maternal multivitamin intake during or between pregnancy (yes/no),
 Dedicated lactation (yes/no),
Infant gender,
 ASD outcome: broad definition of ASD (ADIR score).

The last three research questions addressed whether the relationship of pregnancy exposures (maternal smoke exposure and diet during pregnancy, and lactation) to ASD outcome varies by preconception parental age, preexisting maternal health, or obstetrics. Research Questions 6-8: How does the exposure-timing relationships between pregnancy exposure-timing variables (maternal smoke exposure, diet, and lactation) and ASD vary by preconception parental age, preexisting maternal health conditions, and obstetric risk?

H₀6: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome does not vary by preconception parental age.

H_a6: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome does vary inversely by preconception parental age.

Variables: Prior maternal smoke exposure (yes/no),
 Maternal multivitamin intake during or between pregnancy (yes/no),
 Dedicated lactation (yes/no),

Maternal age less than 36 years; or > 36 years at first birth.

ASD outcome: broad definition of ASD (ADIR score).

H₀7: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome does not vary by preconception maternal health.

H_a7: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome varies positively with preconception maternal health.

Variables: Prior maternal smoke exposure (yes/no),

Maternal multivitamin intake during or between pregnancy (yes/no),

Dedicated lactation (yes/no),

A preconception risk factor (maternal high blood pressure, diabetes,

low iron/anemia, vitamin B deficiency/neural tube risk

[low folate], albuminurea) (yes/no)

ASD outcome: broad definition of ASD (ADIR score).

H₀8: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome does not vary by obstetric risks within the cohort.

H_a8: The relationship between pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD outcome varies positively by obstetric risks within the cohort.

Variables: Prior maternal smoke exposure (yes/no)

Maternal multivitamin intake during or between pregnancy (yes/no),

Dedication lactation (yes/no),

An obstetric complication: (preeclampsia, jaundice delivery) (yes/no).

ASD outcome: broad definition of ASD (ADIR score).

The purpose of this quantitative study was to describe the temporal, hierarchical relationship between pregnancy related exposure factors to ASD; the effect modification of preconception, and confounding neonatal traits that may be associated with ASD and the gender-based diagnosis of autism. Group comparisons between cases and controls, intraclass correlations between genders was conducted while testing for main effects, covariates, and interaction of study variables. Multivariable regression analysis included logistical regression.

Statistical treatment. For each research question, frequency distributions, variable mean, median, and standard deviation were calculated, followed by calculation of difference estimates, bivariate analysis, odds ratio confidence intervals, and log regression multivariable analysis. Data on smoke frequency and breastfeeding duration were converted to ordinal data.

For the first research hypotheses, (research questions one, two and three) logistic regression analysis was hypothetically described in the initial proposal by the following equation for main effect, or pregnancy exposure variables depicted in Figure 2:

$$Y(\text{ASD diagnosis}) = [1.3(\text{smoking}) + 0.8(\text{maternal diet}) + 2(\text{lack of lactation})$$

The above series of hypotheses was tested for the broad ASD outcome definition (ADIR) for the 733 complete records within the AGRE data registry. The most robust regression analysis for relationship of pregnancy exposure to ASD outcome was carried forward to study the potential confounding effects of neonatal traits, and the covariate effect of preconception parental age, preexisting maternal health factors and obstetric

risks. The hypothesized a priori relationship for the overall relationship for exposure-timing factors in Figure 2 was:

$$Y(\text{ASD diagnosis}) = \beta_1(1.3(\text{smoking}) + 0.8(\text{maternal diet}) + 2(\text{lack of lactation}) + 4(\text{infant male gender}) + Z_i(\text{parental age}) + Z_{ii}(\text{maternal preconception health}) + Z_{iii}(\text{obstetric health})) + \epsilon$$

with adjustment for infant respiratory distress

Stratified bivariate analysis was used to determine whether there were possible systemic error sources of confounding, or interaction effects among infant traits and the relationship of proposed main effect variables and ASD. Stratified bivariate analysis determined no systemic confounding effects of neonatal infant traits, including infant gender, shown in Figure 2.

Subsequent logistic regression analysis was proposed to study the primary relationship of prior maternal smoking and multivitamin use, and lactation duration to ASD outcomes and determine the strength of associations via odds ratio analysis. The outcome of broad ASD status, as a binary variable was used to assess the relationship of prior maternal smoking and multivitamin use, and dedicated lactation practice as the main research questions associated with plausible placental transfer mechanisms.

Subsequent logistic regression was proposed to assess the impact of proposed and post-hoc identified covariates to the ADIR score outcome, and the gender differential of ADIR score. Prior cohort studies which reported offspring gender-stratified results in AGRE populations contained 60 to 70 females among sample cohorts of 240 to 300 children

(Carayol et al., 2011; Lamb et al., 2005; Stone et al., 2004 and 2007; Yonan et al., 2003). Parental gender AGRE studies contained up to 993 families (Fradin et al., 2010) or as few as 312 subjects with 175 ASD cases (Cantor et al., 2007). This study used 733 final child records. Data on maternal diet and preconception variables for cohort sample sizes of 200 to 570 children, including 70 and 160 affected females was reportedly available (Eve Landa & Ryan Butler, AGRE liaisons, personal communications, August 22, 2013).

Multiple statistical procedures. For the first hypothesis, bivariate case-control comparisons were used to test the proposed “main effect exposures” of prior maternal smoking and maternal multivitamin use, and exclusive lactation to ASD risk. Then, bivariate analysis between the “main effect” exposures individually or in combination, in Figure 2 and suspected confounding infant gender was tested to adjust for the effect on the relationship of “X” to “Y” outcomes. Odds ratio intervals for ASD risk by main effect variable was reported by gender.

For the second series of hypotheses, the effect of covariates or “Z” variables listed in Figure 2 was conducted to test the impact on the relationship of pregnancy and lactation exposure variables to ASD outcome. It was presumed that preconception parental age, preconception health, and specific obstetric complications mediate the effect of “X” variables on ASD. The hypothesized exposure-timing framework in Figure 2 assumed obstetric complications were predetermined during preconception period. It was plausible that obstetric complications and/or infant breathing traits, may be main-effect or covariates variables associated with the gender differential of autism. Bivariate analysis of hypothesized “W” confounders of infant breathing and sleeping pattern, with resultant

“main effect” variables was to test interactions of variables. The study examined the association between main effect pregnancy and lactation variables to one ASD diagnostic criteria (ADIR, broad definition), stratified by infant gender. Multivariate model predicting the ASD was constructed for the main effect exposures and potential confounders. A series of regression models were fitted to the data, using "enter" stepwise protocols for the resulting optimized main effect and covariate variables.

Matching variables considered the diversity among and familial and nonfamilial control subgroups and the differences were detailed; but suspected and proved to be non-significant. Values of independent variables were collapsed to indices to increase statistical analysis power with recognition that such a technique may have no clinical significance. Logistic regression was used to summarize the odds ratio as a measure of the association between maternal prior smoking, multivitamin use, lactation practice, child gender, and ASD risk.

Because archival, secondary data was used to conduct statistical analysis, approaches were taken to ascertain internal and external data validity. Although the proposed hierarchical framework was premised on temporal aspects of factors, as well a biological plausibility rationale related to placental transfer mechanisms for direct fluid, nutrient and gas exchange for main effect variables, the proposed study variables may not be completely independent variables. But because the variables were collected for general medical record purposes, primarily to supplement genome-wide association test research, it is presumed history and maturation bias is minimal or at least randomly distributed. Other data assumptions are detailed in Chapter 4.

Threats to Validity

The proposed experimental design was a classic design used for rare disorders with less than five to ten percent population prevalence (Gerstman, 2008). The retrospective study design, ability to collapse independent variables to categorical and binary responses, and the use of archival matched control samples were beneficial to efficient implementation of the data analysis. However, case-control designs are subject to sources of bias which affect the ability to generalize the findings to other sample populations (i.e., external validity), and the ability to conclude the relationship of independent variables are actually significant to predicting study outcome (i.e., internal validity). Threats to external validity may be related to the narrow characteristics of the AGRE population, which are predicated on referral by healthcare professionals for families who have two or more members with confirmed clinical diagnosis of autism. The geographical setting of the AGRE registry, in Southern California and the affiliation with nonprofit advocacy groups such as Autism Speaks may not have allowed generalization of conclusions to other U.S. cohorts or autism registries. In this dissertation, comparison of the AGRE sample cohort to U.S. population prevalence rates was conducted to estimate generalizability and external validity of the cohort. In addition, Table 2 describes findings of The M.I.N.D Institute's CHARGE study on autism, which is an independent study population primarily based in Northern California (Ashwood et. al., 2008). The familial-based, genomic focus of the AGRE registry population also based in California is valuable in allowing case-control sampling while controlling for "shared environment" versus unique risk exposures.

Common internal validity limitations of previous studies based on case-control study designs were anticipated and addressed by the proposed methodological framework in this study. Uncertainty bias due to completeness of medical records, dietary recall, genetic diversity, and co morbid conditions are limitations of case-control designs. In this study, accuracy of exposure definitions was increased by using both maternal and paternal age and smoking factors, assessment of maternal anemia during preconception period by self-report as well as physician-diagnosed anemia. Measurement or recall bias of smoking behavior is known to be confounded by maternal education level, other socioeconomic variables, and mode of data collection (Burstyn et al., 2012; Dietz et al., 2011; Kalkbrenner et al., 2012; Lee et al., 2012; Vinikoor et al., 2010; Zhang et al., 2010). Nondisclosure of smoking tended to be higher for young women (age 20-24 years) in a U.S. cohort which quantified blood cotinine concentration to validate smoking status (Dietz et al., 2011). Even when large sample sizes were used in previous studies, cross-sectional, case-cohort and case-control designs commonly did not address gender specific risks associated with maternal smoking and autism.

Retrospective studies may threaten internal validity due to history threats and recall bias, as well as maturation threats (Issel, 2004, Chapter 10). History threats arising from the time passage from maternal pregnancy, to infant delivery and subsequent childhood autism diagnosis may introduce bias which may have affected maternal recall on self-reported survey instruments (Creswell, 2009). For these reasons, the nine study variables were proposed to be grouped and analyzed with regard to temporal aspects of preconception health, pregnancy and lactation period, and infant (0 to 12 months) traits

associated with lactation, sleeping and breathing. Validation of parental age at first birth, with responses for smoking, vitamin use, and obstetric complications was used to increase internal validation. Similarly, maturation bias, due to parental or maternal behavioral or attitudinal change may have occurred from initial enrollment in the AGRE registry (Creswell, 2009). Maturation and testing bias may be particularly relevant since new or supplemental survey questions related to dietary behavior (i.e., maternal fish intake) were administered during the currently proposed study protocol (Creswell 2009). Whether using the archival data sets or supplemental survey data, the risk of information bias or defects in categorical measurements would be minimal if nondifferential misclassification occurred to the same extent in case and control subjects (Gerstman, 2008). However, since many of the matches used in the AGRE sampling frame included familial controls, it is expected and assumed that differential information bias was minimized in the design.

If a newly revised or supplemental survey tool were administered to collect additional or missing data on maternal dietary practices were proposed, this may introduce an interaction of history, and recall during AGRE recruitment with recruitment and participation for the proposed supplemental survey data. Estimation-maximization algorithms for "missing at random", and the more plausible Bayesian "not-missing-at-random" techniques were proposed for missing values. However, despite several missing values, adequate statistical power was achieved ($n=733$).

Statistical regression bias may occur if extreme response measurements were included in group comparison analysis. For these reasons, univariate descriptive analysis

and box plots analysis were used. The use of categorical data intervals used by other AGRE researchers and published in peer-reviewed articles for pervasive developmental disorders and ASD was used to minimize regression bias. The use of multiple logistic regression did not require normal distribution of the independent response measurements (Halpern & Visintainer, 2003).

External validity related to selection was minimized by the use of archival, well-characterized cohorts and blinded data abstraction techniques. But participation in AGRE sampling frame is voluntary and may be restricted to biological families who have two or family members diagnosed with autism spectrum per ADOS or ADIR diagnostic criteria (Lajonchere, 2010). In addition, referrals from clinical and medical professionals were the primary AGRE ascertainment method. Participant attrition bias for families who were referred but did not join AGRE sample cohort and registry is unknown but is assumed to reflect nondifferential bias.

Ethical Procedures

Application to the archival AGRE research system required a Data Access Application, Researcher Distribution Agreement (RDA) and AGRE IRB approval documentation executed by the principal investigator to address legal responsibilities for data use, data sharing, researcher generated data requirements, confidentiality, access, data storage, participant withdrawal rights, and acknowledgement of use of AGRE proprietary, coded archival data. Annual notification to AGRE of researcher generated data, prepublication manuscripts and the like were required and addressed on December 3, 2014 in compliance with the terms of the AGRE agreements.

The focus of this proposal was clinical research data collected by survey instrumentation. No DNA, blood cultures, or other biospecimens were requested from AGRE for this proposal. The scope of the research distribution agreement was for clinical data which excluded any personally identifying information about the family or its members. Clinical variables included age at time of testing, sex, ASD criteria, and family coding, and medical history variables.

Participant and family names and contact information were not recorded or shared with the primary researcher. Unique identifiers were predetermined by AGRE data administrators (Lajonchere, 2010; Johnson et al., 2010). No transcriber or translator services were anticipated or used. Medical records with the AGRE research registry are retained on the OSCR online platform for clinical research data management (Lajonchere, 2010). Data was exported and formatted to Microsoft Excel and/or Access files for descriptive and quantitative statistical analysis using SPSS version 21.0 and EpiInfo™ software without external support.

Results may be shared among AGRE administrators and AGRE registrants pursuant to the terms of AGRE IRB and ethics policies, Data Use Agreement, and AGRE Research Distribution Agreement requirements. Registry participant informed consent was not required for the use of retrospective, coded, blinded, clinical data sets available through AGRE research agreements.

Protection of Private Health Information

Human research protections were partially addressed via existing Walden IRB approvals. AGRE's RDA and IRB approval ensured patient privacy and protected health

information. All patient identifiers were precoded by AGRE's OSCR internal data control system. Therefore I was fully 'blinded' to unique patient identifying information. No private health information was or will be published as part of the proposal, oral defense presentation, thesis or as part of any subsequently drafted scholarly publications. IRB approval number (# 11-14-13-0074350) for clinical research is provided to reference details of data privacy, secure data management procedures, confidentiality agreements, conflicts of interest, Letters of Cooperation, and Data Use Agreements with the Autism Genetics Resource Exchange (AGRE) partner.

Treatment of Data

Approved access to archived medical record datasets obtained from AGRE associated with pre-blinded or recoded patient identification numbers was obtained by the principal investigator on December 16, 2013. Precoded and cleaned patient information was received to protect anonymity of cases, controls, and families. No research assistants or statistical consultants were used, and data storage was limited to one primary desktop computer with a backup external hard drive. All record retention, data security and storage, and confidentiality of information was and will be maintained throughout data analysis, data summary, interpretation, presentation, and publication. Data access required secure, unique authenticated encrypted access to AGRE portal and data exchange systems. Separate computer passwords and hard disk media were not available to another household member. Ethernet computer was used for data exchange with AGRE. Data file formats, field and variable specifications within Microsoft Excel were

aligned as necessary to configure files for SPSS data analysis. Data presentation included aggregated measures to the extent individual patient records cannot be inferred by results.

Summary

The case-control study design, modeled after the hierarchical framework of Burstyn et al. (2011) reflected the current thinking about temporality of risk factors of ASD, and the gender-differential of ASD. The proposed framework reflected temporality of exposures, genetic susceptibility, and placental transport theories. Methodology was proposed to describe the AGRE cohort sample and archived data. The sampling frame, matching protocols, and categorical treatment of variables were discussed and aimed to replicate approaches of prior ASD research, and AGRE research protocols. Data sources resulting from retrospective administration of standardized AGRE medical survey instruments were obtained through appropriate data access and IRB approvals. Data dictionaries, data coding and manipulation to binary values, indexed values for obstetric, preconception variables was considered, as was univariate, bivariate analysis, and methods to identify confounding and interaction terms.

Hypothesized regression analysis equations were proposed to weigh the main effect variables of maternal prior smoke exposure, multivitamin use, and lactation to ASD. These relationships were analyzed as proposed for the broad ASD outcome (ADIR score). Data coding was conducted to reduce variables to binary values for logit regression analysis. Effect modification of gender, and preconception and obstetric health risk factors were proposed and analyzed as initially proposed. Threats to validity were

identified, and limitations of generalized conclusions based on a case-control design as a hypothesis testing approach were acknowledged.

I explored the relationship of maternal diet and prior maternal smoke exposure and lactation as main pregnancy exposure factors of subsequent ASD diagnosis in offspring. However, it is understood that statistical correlation may or may not be related to causation. Three major reasons for this may be because; 1) underlying mechanisms are not linear functions, 2) the attributable variables are not continuous in nature, and/or 3) spurious and simultaneous monitoring of two continuous variables may be each described in linear functions without be associated or linked to each other (Gerstman, 2008). In addition, the relationship of the independent exposures may or may not be temporally related to each other, or to ASD risk.

Chapter 4: Results

Introduction

The purpose of the study was to use archival secondary AGRE data sets to describe the exposure-timing relationship between pregnancy traits (maternal smoke exposure and diet during pregnancy and lactation) and ASD outcome as mediated by preconception parental age, maternal health, and obstetric complications, and as confounded by neonatal traits. I aimed to contribute to the limited body of research on the hierarchical relationship of temporal factors, shared familial environmental risks, unique exposures and risk factors, and infant gender to the risk of ASD. Pregnancy factors theorized to represent preconception health and placental transfer mechanisms were assumed to be the main effect risk factors affecting ASD offspring.

Research Questions and Hypotheses

The research questions addressed exposure-timing relationships of pregnancy and ASD outcome and ASD gender differential, as adjusted for hypothesized confounding neonatal factors and covariates of preconception risk. Based on the independent variables of maternal smoke exposure and multivitamin use, lactation, and gender and their combination, subhypotheses were proposed. Covariate preconception factors and confounding infant traits were analyzed independently to inform the relationship to ASD and ASD gender risk.

The initial research questions addressed the following: What is the exposure-timing relationship between pregnancy factors and ASD outcome within the AGRE cohort? Secondly, the question of whether the relationship of pregnancy factors to ASD

outcome is confounded by neonatal traits was explored. The third series of questions tested whether preconception factors are effect modifiers of the relationship of pregnancy factors and ASD.

The framework for the study was presented in Figure 2. Archived data discrepancies, variable definitions, and coding will be described in this chapter. Data access, data collection, and record collation from various AGRE databases also will be explained. Data transformations, statistical univariate and bivariate analysis, and comparative results with other AGRE cohort studies are included. Statistical power analysis will be discussed with regard to feasibility of regression analysis. Proportional differences and odds ratio analysis is also detailed.

Data Collection

Access to the AGRE phenotypic data registry was obtained and copies of individual files ("Affected Child, Unaffected Sib, Mother History, Father History, Metals and Mothers") were downloaded as Microsoft Excel files. Fragile X database (revision 030409 with 1139 records), ADOS-G, Module 2 (2000 and earlier, with 588 records), and ADIR (2004b version with 3718 records) were used to cross-check columns in "Affected Child" and "Unaffected Sib" databases. An overview of AGRE survey instruments and associated record counts is shown in Figure 3. Raw Affected Child records ($n = 732$) were inspected and case exclusion criteria for Fragile X, Cystic fibrosis, Down's syndrome, and Wescler's Intelligence Quotient score less than 70 were applied, resulting in the exclusion of 20 records. The Affected Child file included offspring from 350 families or AU-family codes and included 16 control (ADIR = 0) families with 37 child

records (27 boys, 10 girls). Unaffected Sib records ($n = 194$) were inspected for exclusion criteria, and two records were excluded due to inability to test for ADIR. After merging the Affected Child and Unaffected Sib files, there were 606 ASD cases, as defined by ADIR score of 1 (477 males, 129 females) and 296 controls (ADIR = 0).

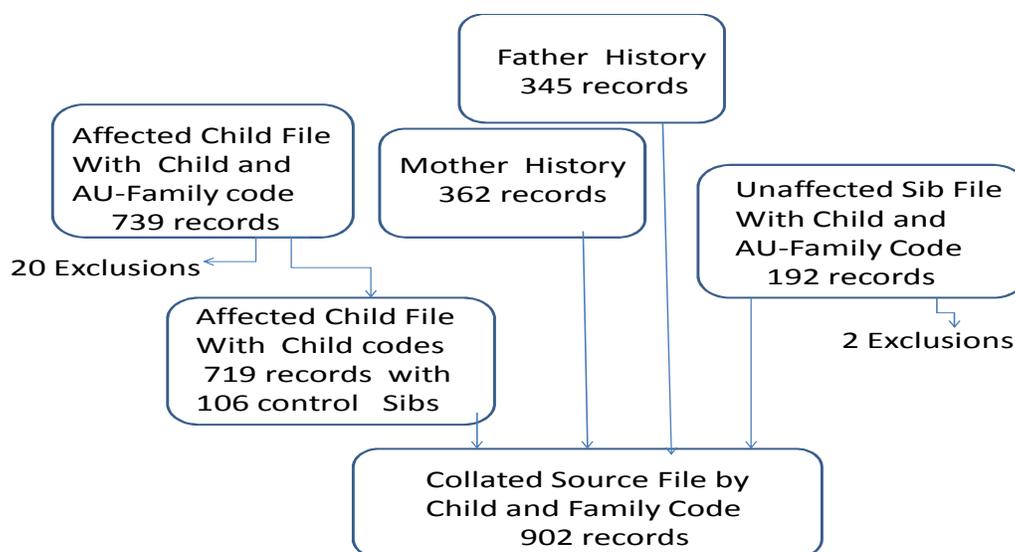


Figure 3. AGRE databases, survey instruments, and record counts.

The AGRE control group had 132 females and 164 male offspring. Records for Unaffected Sib and other nonfamilial controls were often incomplete for the a priori predictor variables when originally obtained.

Separate survey instruments reflecting maternal and paternal health history ("Mother History" and "Father History") were recorded at a similar enrollment period as the survey responses for Affected Child and Unaffected Sib surveys. Parental history file records were not specifically coded to an enrolled child record, but the affected child identifier could be deduced from the family code and age of parent at AGRE enrollment. Maternal and paternal history responses on "prior" and "ever" smoking practice were

used to cross-check maternal preconception risk and smoke exposure responses recorded in the Affected Child data file.

Survey instruments had redundant or repeated questions regarding lactation duration, maternal smoke exposure, and maternal preconception and obstetric risk factors. In these instances, supporting databases, such as "Metals and Mothers," were used to test consistency of Affected Child records or supplement the Affected Child and Unaffected Sib child records. Lactation practice and duration data were supplemented for 12 control records by cross-referencing data included in the Metals and Mothers dataset, which included lactation data.

Discrepancies from the originally proposed data plan included ASD outcome definition criteria, at least 40% of records missing maternal variable values, and proxy survey items related to temporality and specificity of exposure of hypothesized main effect variables (i.e., "prior, ever, current" smoking and "any" or "dedicated" lactation) are described in this section. Baseline descriptive traits of the AGRE sample are also described in this section. Internal data consistency, data recoding, data transformation, treatment of missing values, tests of normal or nonparametric distributions, univariate analysis, and covariate adjustments are also described.

Data Discrepancies and Proxy Variables

Case definition. The AGRE case definition of ASD within the Affected Child file was considered ASD "affected" for records in which either an ADIR (Autism Diagnostic Interview-Revised) or ADOS (Autism Diagnostic Observed Schedule) score was greater than 0 (Western Psychological Services, 2010). ADOS Module data were available for

Modules 1, 2, and 3 for young children, aged less than 12 years and verbal fluency for children older than 16 years, respectively (Western Psychological Services, 2010). Only 30% of Affected Child and Unaffected Sib records contained both ADOS-3 and ADIR scores. Among those records, 20 records were ADOS positive with ADIR scores of 0. Nine records were ADIR positive with ADOS score of 0. Because more than 70% of Affected Child records had no ADOS-3 score value, only ADIR criteria were used as case criteria. No Affected Child records had missing ADIR data. File matching with ADOS-G Module 1 database did not resolve the issue and contributed no additional records with the variables of interest in this study. ADIR scores of 0 (control or unaffected sibling) and ADIR score of 1 (ASD case positive) were used as the outcome variable in all statistical analysis of ASD status.

Exposure parameters. Variable definitions were largely available as proposed, but temporality and specificity of exposures were clarified during data coding and manipulation to closely approximate a priori variable definitions and align with the conceptual framework. Clarification of temporality of smoking frequency and lactation duration required recoding to binary responses to exclude overlapping exposure periods for questions in the Affected Child and parent history ("Mother History", "Father History") survey questionnaires. Figure 4 depicts temporal response data and coding used for smoking and lactation behavior to establish narrowed definitions of prior (preconception or pregnancy) maternal direct inhalation smoking reported in parent history questionnaire, and dedicated lactation practice (not including casual breastfeeding or complimentary breast and bottle feeding).

Maternal smoking was characterized by the responses to prior to enrollment maternal smoking behavior as reported by the mother or father in the Mother History or Father History survey instruments. Prior smoking was further refined to distinguish Prior Mother, Prior Father, or Prior Both (Parent) smoking exposure type. Maternal smoking data were further analyzed for prior direct smoke inhalation by mother versus father. In the case of twins or multiple births within the same pregnancy, maternal smoking behavior exposure was assumed to be similar for all births within the single pregnancy.

Supplemental information on "ever"

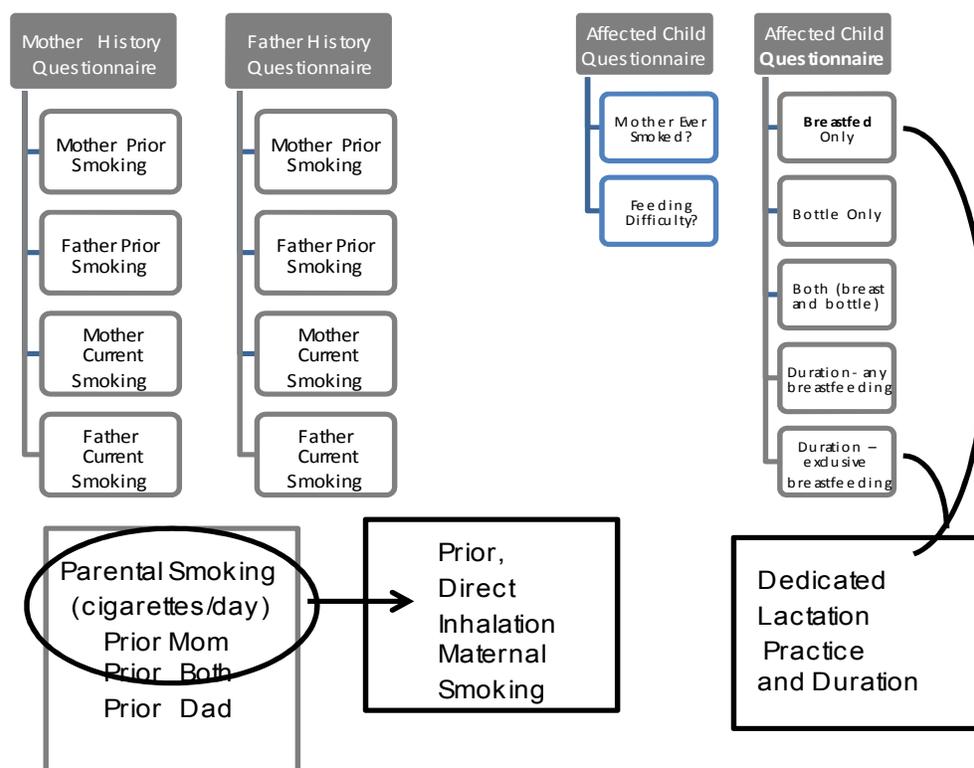


Figure 4. Variable definition and recoding of data for smoking and lactation questions.

smoked was available from the Affected Child database but was not used as a primary data source for direct smoke inhalation measures of maternal smoking. Smoking frequency was recoded from continual, open-text response values to three a priori ranked categorical values. As the resulting categories exhibited multiple modalities, the three categories were expanded to five categories.

Maternal diet factors included data responses to items on the Affected Child database survey instrument for multivitamin use before or during pregnancy as the primary data source. Responses were inspected for completeness and validity with regard to a separate survey question about over the counter medicinal use by the mother before or during pregnancy recorded in the Affected Child database survey instrument. Very limited amount of data were available on fresh fish intake as indicated in the Metals and Mothers survey instrument. This database provided only supplemental information for a very limited number of records within the AGRE sample, and the data were collected approximately 10 years after child AGRE enrollment. Response values were retained as binary data for multivitamin use and fish intake.

Lactation questions within the Affected Child database survey questionnaire recorded responses to any lactation, combined breast and bottle feeding behavior, and dedicated lactation practice. Responses were checked for internal consistency and breastfeeding practice was redefined to quantify only dedicated and nondedicated lactation practice duration. Durations were reported as open-text field variable responses, typically in units of months.

Lactation data were checked against responses in the Metals and Mother survey instrument. Lactation data were recorded as similar for multiple birth pregnancies unless otherwise indicated by responses to questions in Affected Child or Unaffected Sib surveys. Lactation duration was recoded from open-text response values to four a priori ranked ordinal categories. Since the coded lactation data showed multiple modalities, the categorical data for lactation duration was expanded to five more representative ranked categorical values.

Parental age was recorded in the Affected Child database as the age of mother or father at AGRE enrollment. When parental age was missing or blank in the Affected Child database, it was obtained from the Unaffected Sib database by deduction of other childrens' ages. Parental age was analyzed as a continuous variable and analyzed after log transformation.

Preconception risk factors were previously defined for this study to include pre-existing diabetes, high blood pressure, anemia, albuminuria, and low vitamin B/folate/neural tube risk. Preconception risk factors were recorded in response to survey questions asked within the Affected Child and Unaffected Sib databases. These values were cross-checked with Mother History records, but the responses for Affected Child instrument were the primary data source. AGRE responses for maternal triple screen test were used as a proxy variable for exclusion criteria for Down's syndrome, risk of spina bifida, or low vitamin B/folate in instances where these diagnoses were recorded to be the outcome of triple screen abnormality. Total preconception risk was tallied for binary responses to the above five preconception risk factors. Preconception risk score was

assumed to be similar for multiple fetuses (twins, triplets) unless otherwise indicated by comments in the AGRE databases.

Obstetric complications were previously defined as preeclampsia and hyperbilirubin or jaundice by self-report within the Affected Child instrument. Binary responses for survey questions for these risk factors were obtained as proposed and required no adjustment or recoding. Obstetric risk was tallied for binary responses to the aforementioned risk factors. Obstetric risk factors were assumed to be child specific and not similar among multiple births.

Proposed covariates of infant breathing and sleeping disorders were defined by proxy variables. Respiratory distress and resuscitation at delivery were retained as separate variables. Due to the conceptual framework based on SIDS or hypoxia related risks associated with ASD, infant (0-11 month) breathing traits obtained from Affected Child and Unaffected Sib instruments were used as primary data source variables. Data for infant respiratory distress at birth and infant resuscitation at delivery by parental self-report recorded as binary responses in the Affected Child and Unaffected Sib survey questionnaire items were analyzed posthoc. Infant respiratory distress and resuscitation was not assumed to be similar among multiple births. Responses for infant traits were retained a priori and analyzed as binary response variables.

Maternal smoke exposure. Parental report of smoking prevalence within "Mother History" and "Father History" files were used as the primary data source for several reasons. These reasons included specific temporality, discrimination of prior/maternal smoking behavior from indirect maternal smoke exposure, and more descriptive

responses from parental history records. Smoke exposure temporality and open-text responses for smoke frequency required recoding. However, there was good agreement for nonsmoking prevalence by questionnaire.

“Current” and “prior” smoking behavior questions were asked in the parental survey instruments at date of AGRE enrollment, typically when the affected child average age was approximately nine years old (see Figure 4). Parental history comments about “prior” smoking often referred to tobacco smoking, during teenage or college years, or prior to the affected birth. “Prior maternal smoke exposed” was defined as prior tobacco use by the mother or both parents. For the Affected Child survey instrument, the question was phrased broadly as "ever" exposed for "maternal exposure to smoke". Approximately 52-54% of parents reported no prior smoking behavior among cases and controls. Response prevalence for non-smoking was similar regardless of survey instrument (i.e. Mother History, Father History or Affected Child surveys).

Smoke behavior (binary data) tended to be more frequently reported, and smoking frequency (packs of cigarettes per day or week) responses were more frequently reported in each of the separate parental history files as compared to the Affected Child file (see Figure 5). Smoke frequency was recoded from raw units of packs per day or week, to cigarettes per day, with an assumption of 20 cigarettes per pack. The definition of smoking “like a chimney” was assumed to be equivalent to two packs per day, and “occasional” or “moderate” smoking was assumed to be five cigarettes per day (American Cancer Society, 2014). “Socially” smoking was assumed to be an estimated two cigarettes per day (American Lung Association, 2014).

Data were retained separately for maternal and paternal smoke behavior (binary variable), and smoke frequency for "current", or "prior" smoking behavior(s) for each parent; and for "ever" smoking was retained for each child record. Table 5 shows the raw data smoke responses by survey form. The Affected Child questionnaire data results may suggest under-reporting of smoke frequency (only 32 quantified exposure responses) relative to the reported quantified smoke frequencies reported in Mother History, and Father History files (86 and 112 prior smoke frequency data points, respectively). Therefore, 198 of 271 parents provided quantified smoke frequency results when asked about smoking behavior in the parental survey questionnaires, but only 32 reported quantified smoke frequency information when asked about smoking behavior in the Affected Child survey at enrollment. Self-reported smoking data were more robust within parental history files and justified the use of these databases as the primary data source.

Smoking prevalence, temporality, and frequency information was available for over ninety-five percent of parents of case records, but was available for only half of parents of control records. The lack of information among control records was likely associated with the fact that smoking behavior was not asked as part of the Unaffected Sib survey instrument.

Table 5

AGRE Surveys, Data Records and Coded Smoke Frequency and Means

Questionnaire	Affected Child survey results		Mother History (MH1) with matched Father History (FH1) data			
	Maternal PRIOR		Maternal PRIOR		Maternal CURRENT	
Smoke exposure temporality			MH1 File	FH1 File	MH1 File	FH1 File
Number of Records	712		362	345	362	345
Family Codes	350 families		350 pooled family codes		350 pooled family codes	
Percent Admitted Smokers	38.20%	N= 712	18.5% of 712 child records		43% of 712 child records	
	---- 148 families had Prior AND Current smoked ----- ---- 130 families had Prior maternal smoke exposure -- ----- 47 families had Prior OR Current smoked ----- 330 moms of Affected children had "Ever" smoke data					
			MH1 File	FH1 File	MH1 File	FH1 File
Smoke Frequency for records, n	n =32	N= 271	86	112	39	55
Aggregate Smoke Frequency (cigarettes/day)						
Mean (S.D)			12.6 (7.8)	17.7 (14.1)	6.9 (8.3)	12.8 (11.3)
Median	5		10	20	10	20

Note. Data manipulation: CODED Prior Smoke Exposure defined as Mean of MH1 and FH1 PRIOR smoke per record
 CODED Ever Smoke Exposure defined as Mean of MH1 and FH1 PRIOR AND CURRENT exposure per record

A comparison of the "ever" and "prior" smoke frequency responses, by survey instrument is shown in Figure 5. The data reflected multimodalities. The overall average reported smoking frequency in Affected Child survey instrument was 7.5 cigarettes/day, with a median value of 5 cigarettes/day. Within the Mother's History file, the average and median reported "prior" smoke frequency was 12.6 and 10 cigarettes per day, respectively. Within the Father History file, the average and median "prior" smoke frequency was 17.7 and 20 cigarettes/day (see Figure 5). Fathers tended to report higher maternal smoking frequencies than mothers or responders to the Affected Child survey.

Parental history records were more complete than Affected Child frequency data, and allowed cross-checking of exposure parameters with other datasets, and provided the temporality of preconception risk that best fit the study's conceptual framework.

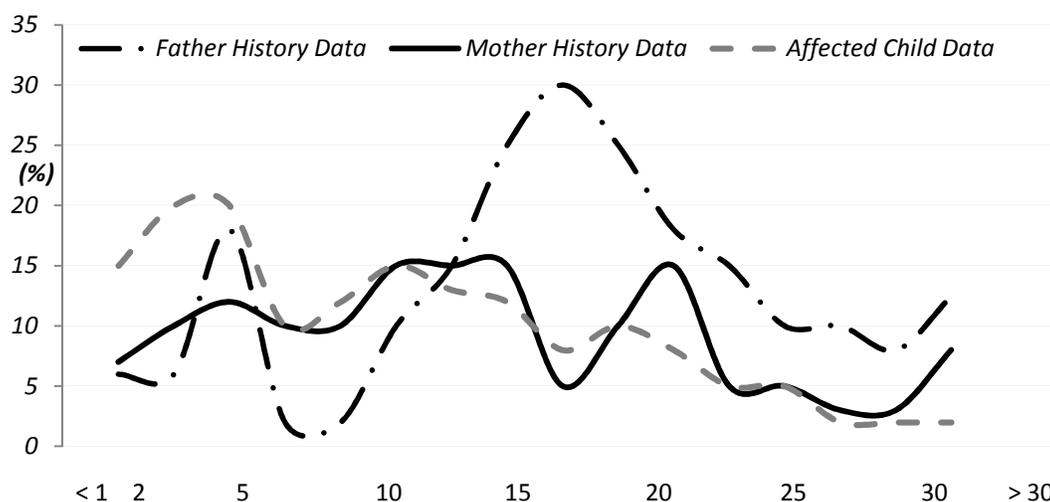


Figure 5. Average daily smoke frequency by AGRE questionnaire (cigarettes/day). Percent of 32 "ever", 86 maternal, and 112 paternal "prior" smoke record results

Maternal prior smoke frequency was not missing in case records, but was missing for 62 of 161 mothers in the control group who reported prior direct smoking behavior. Thus prior parental smoking responses were collected for 82.6% of the sample, but only 73.3% of records had quantified smoke exposure frequency values associated with the prior smoking behavior. While not a key study variable, the quantified responses for "ever" was subsequently coded to reflect the average value of "current" and "prior" frequency as recorded in the Mother History or Father History file (see Table 5). Maternal ever smoking frequency were missing for 27 of 606 case records, and missing for 196 of 296 controls. Analysis for ever smoked frequency was not a study focus.

Comparative distributions for Affected Child, Mother and father History survey responses for maternal smoke exposure are shown in Figure 5 and show wide discrepancies. This variability may be related to the question wording of "current" and "prior", response scale ambiguity (open-text fields), history and maturation bias, and/or recoding misclassification.

The parent identifier code was retained to conduct additional analysis for mother direct inhalation (prior maternal) behavior or indirect smoke exposure (father only smoking). The final, more restricted definition of "maternal prior smoke exposed" for this study excluded prior father-only smoke exposure, since the proposed relationship was based on directly attributable placental transfer mechanisms. A maternal direct exposure definitional criteria was imposed in an attempt to minimize statistical type I error rate.

Figures 6 and 7 illustrate the maternal smoke exposure frequency distributions for mothers who were previously exposed to smoke (prior smoking by mother, or both parents), or ever exposed to smoke exposure. Overall, prior smoke exposure profiles tended toward higher non-parametric mean and median values than ever smoke exposure smoke frequencies (cigarettes per day). No distribution had outlier values above the range of (Quartile 3 plus 1.5 times the interquartile range) by gender or ADIR status; but all distributions were multimodal.

Graphs in this section illustrate maternal smoke behavior and exposure frequency based on a standardized distribution of percent of the sample by ADIR status. The duration scale is not linear in Figures 4 and 5, but reflected natural clusters in the data responses for the open-text response field. Parents commonly responded in terms of

fractions of cigarette packs which may reflect why raw data responses appeared to cluster by interval. Standardized coding of "social smoking", "a few", "moderate" may also have contributed to the apparent clustering of smoking frequency values. Analysis of smoke frequency was summarized by ordinal categories.

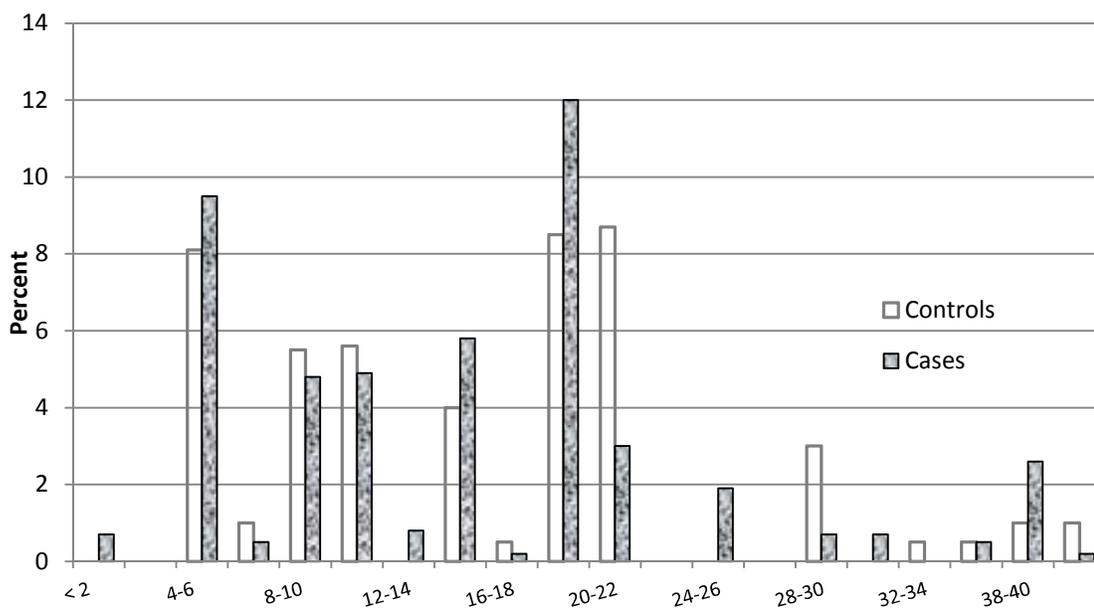


Figure 6. Maternal prior direct smoke exposure in cigarettes per day (n= 681; 99 controls, 582 cases. Non-smokers: 53% controls, 54% cases)

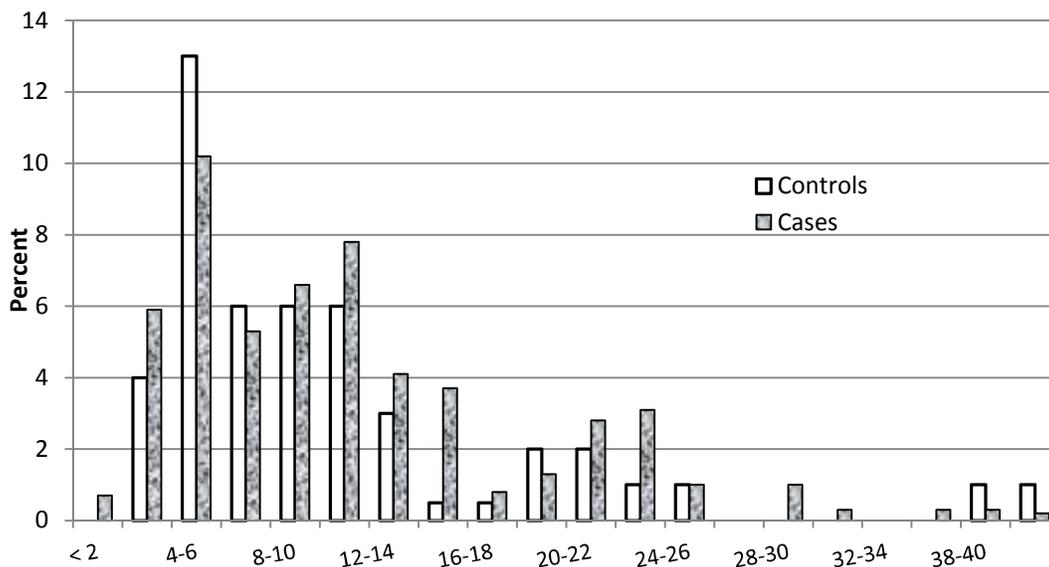


Figure 7. Maternal ever smoke exposure frequency in cigarettes per day (n=679; 100 controls, 579 cases. Non-smokers: 53% controls, 54% cases)

Nonparametric smoke frequencies distributions were inspected to test the a priori assumptions for categorical treatment for smoke exposure (none, < 10 or ≥ 10 cigarettes per day). Data in Table 6 shows the distribution of maternal prior smoke exposure and maternal ever smoke exposure frequency for the entire sample. The results showed maternal prior direct smoke frequency had higher mean/medians than the ever smoked distributions. Table 6 results also suggest the a priori assumptions regarding categorical treatment of maternal smoke exposure may need to be more refined to distinguish effects of maternal smoke exposure for average frequencies beyond 10 cigarettes per day. No values were deemed statistically extreme.

Table 6

Maternal Smoke Exposure Frequency in the AGRE Cohort

Cigarettes/day	Less than five	Five to ten	Ten to twenty	Twenty or more
Ever smoke, %	9.7	37.8	41.4	11.1
Prior smoke, %	1.3	34.2	43.7	20.8

Note. Sample size for Ever exposure was 679. Sample size for Prior direct exposure was 681. Nonsmokers represented 53-54% of the AGRE samples as shown in Figures 6 and 7.

Maternal vitamin use. Prenatal vitamin use data were obtained primarily from Affected Child responses. Limited data on fresh fish maternal intake was obtained from Metals and Mother survey responses. Prenatal vitamin use information was widely available whereas prenatal fresh fish intake was less than 4% complete. Questions about prenatal multivitamin use were asked during AGRE enrollment, whereas the subsample data of the AGRE cohort used to collect maternal fish consumption before and during lactation (Metals and Mother survey) was collected approximately eight to ten years later (in 2010 - 2011) using an overlapping but different sampling frame that that used for Affected Child and Unaffected Sib survey instruments. Therefore, the information available for prenatal fresh fish intake was found to be non-representative and was not used in subsequent statistical analysis.

The AGRE data records collected at enrollment of Affected Child recorded maternal vitamin use by trimester, over multiple trimesters or “all of the above” duration periods. The multivitamin duration value was missing in 54% of Affected Child records, but for recorded durations, the “all” periods was the most frequent response. Multivitamin use at any time was coded as a positive binary response. Less than five

percent of the sample had missing responses for prenatal vitamin use coded in this binary way, and maternal vitamin use was missing in only 6.4% of control records. Dietary supplement use was asked as a separate open-text question in the Affected Child questionnaire. If the parent reported maternal multivitamin use to that question, the data was used as validation or complimentary information to the primary survey question about "multivitamin use". This protocol was used if the data file showed maternal supplementation was recorded in the comment section for the dietary supplement question. It was recorded for six records as a positive multivitamin use response. Prenatal maternal vitamin use data (yes/no) for 227 control and 606 case records were retained for regression analysis.

Lactation factors. Lactation questions included survey items coded for binary responses about feeding practice, a non-mutually exclusive response to a question about breast, bottle feeding or complimentary breast and bottle feeding, and questions about the duration of coded "any" level of lactation as well as "dedicated" lactation duration; each as separate survey questions. Duration of "any" lactation included responses for breastfed-only infants, mothers who used both breast and bottle feeding practice, and mothers who may have attempted or initiated lactation but transitioned to bottle feeding only. Feeding behavior variable or lactation practice was originally defined and recorded within AGRE data sets as "breastfed, bottle fed, or both" with less than 0.5% of case records missing values for lactation practice, but was missing in 151 of 296 control records. In instances of conflicting data on lactation practice, the primary data source was the Affected Child file response information, including open-text field comments in the

survey. After cross checking with lactation duration survey responses and converting the responses to the binary code to define a "dedicated" or nondedicated lactation practice, 296 controls and 606 cases had information on dedication to lactation practice.

Among all cases "bottle only" was reported for 24% and "breast only" response was reported to be 56%. For control records, "bottle only" feeding practice was reported to be 30% whereas "breast only" was reported for 40% of mothers. The coded definition of "dedicated lactation" extracted from all survey instruments was less restrictive than the WHO definition of exclusive breastfeeding which is defined as:

no other food or drink, not even water, except breast milk (including milk expressed or from a wet nurse) for 6 months of life, but allows the infant to receive ORS, drops and syrups (vitamins, minerals and medicines). (WHO, 2001)

Maternal AGRE self-reported lactation practice questions were in reference to bottle-fed infant formula, breastfed only, or both without consideration of other infant dietary offering or intakes.

Duration of lactation was recorded for "dedicated " or "any" periods, using open-text AGRE response fields. Therefore, 602 cases and 158 controls had information on lactation duration. Quantifiable lactation durations were asked as separate questions that could be related to lactation initiation, lactation duration, bottle feeding, or both breast and bottle feeding practice. Lactation capacity, use of weaning foods, or other beverages was not addressed.

Lactation was recoded as "dedicated lactation" for "breast only" responses to the question of infant feeding practice for the variable labeled "breast_bottle_feed".

Lactation practice, degree of dedication, and duration by child record were tested for internal consistency and recoded if non-zero value lactation duration was recorded (in months) or self-report comments included “breast” or “bottle fed” comments within Metals and Mothers survey. The recoding and cross-checking resulted in 602 cases and 151 controls having complete information for dedicated lactation practice, *and* dedicated lactation duration (in months).

Overall, "any" lactation durations recorded for 744 records, skewed toward higher non-parametric values than "dedicated" lactation duration in months for 739 records. None of the combinations of any or dedicated lactation distributions had outlier values above the extreme range (Quartile 3 plus 1.5 times the interquartile range) by gender or case/control status. All reported lactation duration distributions (any and dedicated) were multimodal.

For mothers who reported dedicated laction ($n=402$), the separate survey questions about any duration of lactation and dedicated lactation duration were redundant and were expected to provide similar results. A comparison by subgroup over 18 duration intervals showed an overall difference of 0.6 months for any lactation duration compared to dedicated lactation for mothers who breastfed only. This difference showed a random variation within the same 18 duration intervals used in Figure 8. Random variation was also shown between genders, cases and controls. Thus, the mean duration for each child record was used in instances when mothers reported no bottle use during lactation for both any and dedicated lactation practice. Data were graphed as percentages to better illustrate the distribution and duration ranges.

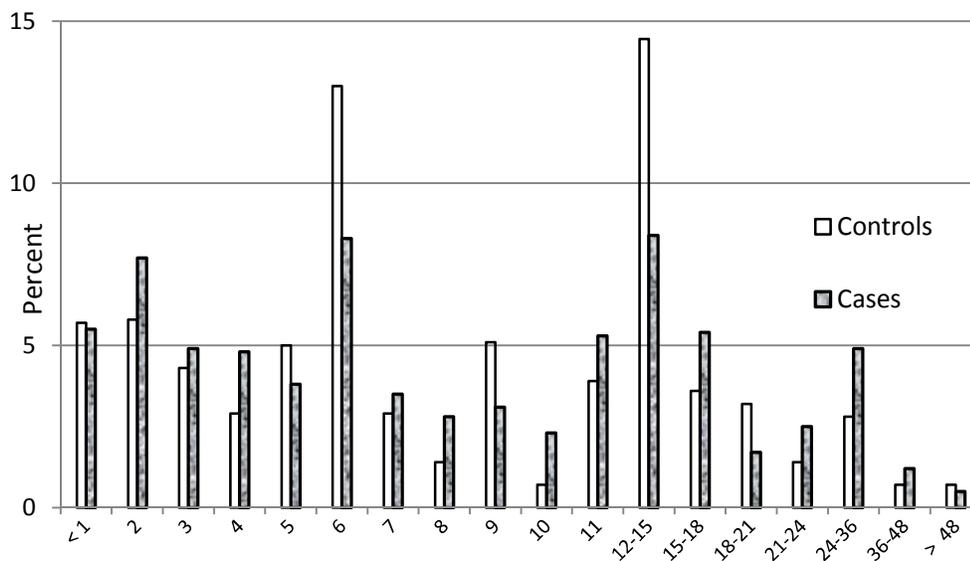


Figure 8. Any lactation duration distributions for AGRE sample (months) (n=744; 138 controls, 606 cases. Bottle-fed: 30 % of controls, 24% cases)

The profiles represent lactation duration among mothers of all 606 cases and 138 controls (of 296 control records); and reflect data for 574 males and 170 females. The duration scale is not linear beyond twelve months duration but reflected natural clusters in the raw data responses for the open-text field of continuous response scale. It is possible that mothers reported lactation duration in terms of fractions of years (and specific temporal periods such as six-weeks maternity leave) which may reflect why raw data responses appeared to cluster by interval.

A narrowed definition of dedicated lactation was proposed a priori and the binary coded data is shown in Figure 9; these data were available for 332 cases and 70 controls (317 males and 85 females). The data showed naturally clustered, nonlinear intervals of

dedicated lactation practice, similar to the interval categories seen in Figure 8 for the "any" lactation variable.

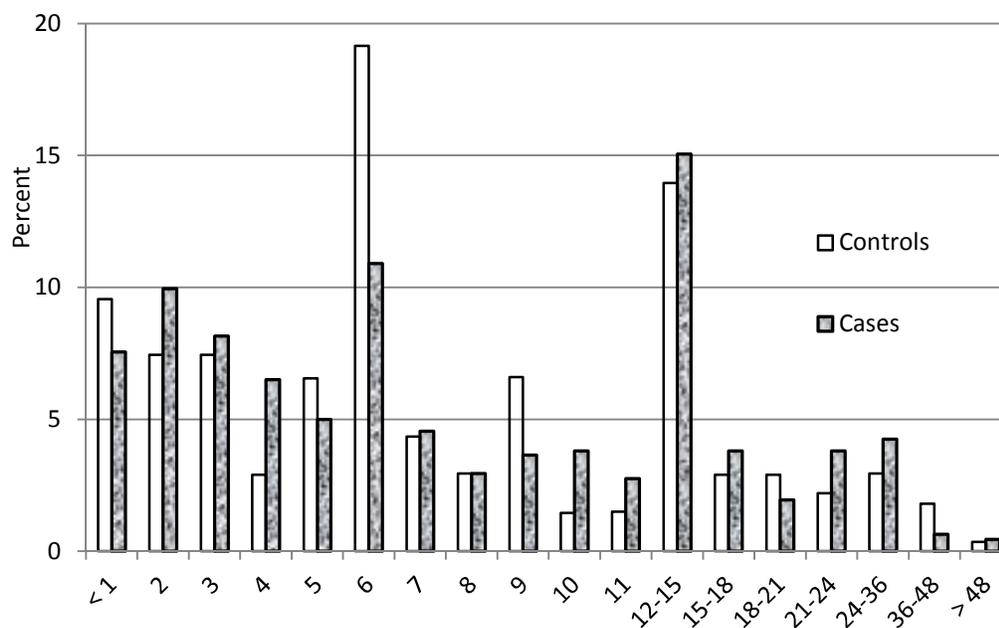


Figure 9. Dedicated lactation duration (months) (n=402; 332 cases, 70 controls.)

Based on their non-normal distributions, both lactation duration variables (any and dedicated) were re-coded into ranked ordinal categories. The distributions were then inspected to test the a priori assumptions for categorical treatment of lactation duration (none, less than 2 months, 2-6 months, and greater than 6 months). Results in Table 7 indicate the a priori assumptions regarding categorical treatment of lactation duration may need to be more refined to distinguish effects of lactation durations beyond 6 months. Since none of the dedicated lactation durations were statistically extreme (i.e.

higher than the value of quartile 3 plus 1.5 times the interquartile range), all values used to create Table 7 were retained for analysis.

Table 7

Distribution of Lactation Duration in the AGRE Sample

Feeding practice and duration (months)	Two or less	Two to six	Six to twelve	Twelve or more
Any lactation, % <i>n</i> = 744	13.9	26.9	41.8	17.4
Dedicated lactation, % <i>n</i> = 402	17.2	29.6	28.0	25.2

Note. Any lactation mean was 6.4 months. Dedicated lactation mean was 4.6 months.

Parental age. Parental age was recorded in the affected child database as the age of mother or father at the time of AGRE enrollment. When parental age was missing or blank in the Affected Child database, it was obtained from the Unaffected Sib database. A similar method was used by previous AGRE researchers (Annello et al., 2009) for 393 matched sibling pairs. Paternal age was missing for 4 of 296 control records, and no ASD case records (*n* = 606). Maternal age at parturition was not missing in any child records.

Maternal age was skewed above the mean values as shown in Figure 10 (*mean* = 31.3 years, *SD* = 6.8). Paternal age distribution was also skewed positively above the mean value and on average was higher than mean maternal age (*mean* = 33.6 years, *SD* = 8.3). Log transformation improved normality of the parental age distributions for the full cohort but subsequently such transformation did not affect regression analysis (data shown in Chapter 5).

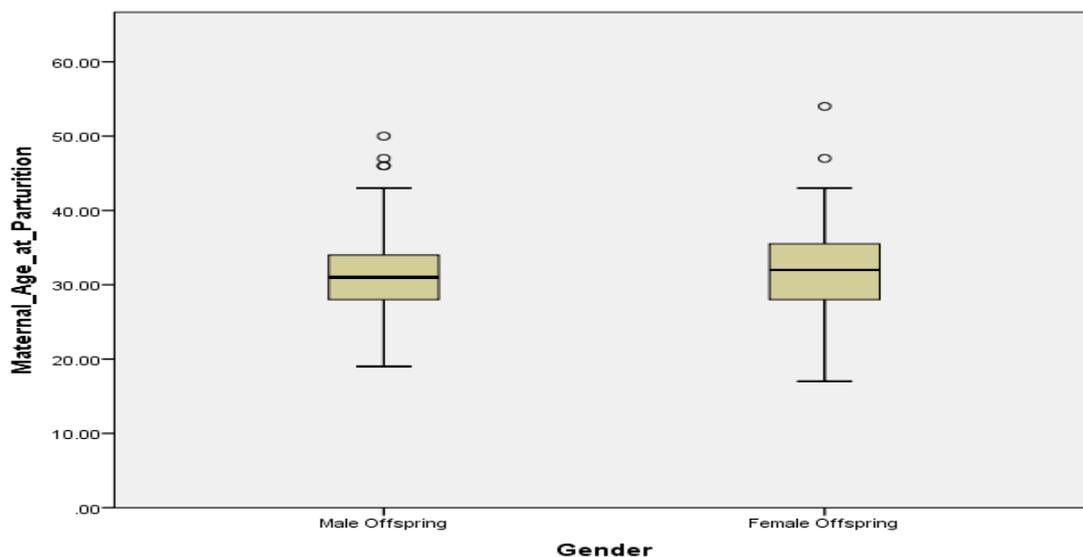


Figure 10. Box plots of maternal age at parturition by gender for all birth types

Preconception risk factors. Preconception risk factors were previously defined for this study to include preexisting diabetes, high blood pressure, anemia, albuminuria, and low vitamin B/folate/neural tube risk. Preconception risk factors were cross-checked using Maternal History records. The survey questions for most of these risk factors were available as originally proposed and requested from AGRE databases. Therefore minimal recoding was necessary for preexisting diabetes, high blood pressure, anemia, and albuminuria. Separate AGRE survey question data was not available for low vitamin B/folate/neural tube risk, but the data results for maternal triple screen test, which tested for alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG), and estriol (uE3) were available in the affected child database. This triple marker tests is a prenatal screen typically done at 15 to 20 weeks gestation to test for birth defects including Down's syndrome, spina bifida, and anencephaly (American Pregnancy Association, 2006). Therefore, AGRE responses for maternal triple screen test were used as a proxy variable

for exclusion criteria for Down's syndrome, risk of spina bifida or low vitamin B/folate in instances where these diagnoses were recorded to be the outcome of triple screen tests.

Within the AGRE case records, binary values for “maternal triple screen test?” had no missing values. The follow-up question on whether the triple screen test result was abnormal for ASD cases also had no missing values. Therefore, abnormal triple screen was a proxy variable for low vitamin B/folate, and albuminuria if noted in the record. Among controls, binary values for "maternal triple screen test" and the follow-up question on triple screen abnormality were missing for 65% (192 of 296) controls records. Among controls, excluding missing values, 36.5% were triple screen tested and 1% of records indicated abnormal triple screen tests. Among cases, 41.3% were triple screen tested and 2.6% indicated abnormal triple screen results.

The most common comments for abnormal triple screen test (38 of 62 open-text-field comments) mentioned kidney or albuminuria diagnosis. In these instances, if albuminuria was not indicated elsewhere in the record, an abnormal triple screen was used to indicate either albuminuria or low folate as a (one) positive response to the preconception risk factor index. Abnormal fetal testing results were presumed to indicate a positive preconception risk response, regardless of amniocentesis status, as less than one third of abnormal triple screens were reportedly followed up with amniocentesis testing. Diabetes and anemia were rarely reported.

Crosstab descriptive frequencies indicated 78% of mothers had a score of "0", 16% had a preconception risk score of "1" and 6% had an overall preconception risk score of "2" or "3". Crosstab statistics by ADIR status indicated total maternal

preconception risk scores had fewer than five counts for the highest rank score category, so the preconception risk scale categories were recoded and collapsed to reflect an ranked ordinal score of "0, 1, or > 1" (data not shown).

Obstetric risk factors. Obstetric complications were previously defined as preeclampsia and hyperbilirubin or jaundice by self-report. AGRE binary survey questions for these risk factors were obtained as proposed, and required no adjustment or recoding. Obstetric complication values for case records had less than 2% missing values whereas control records had 65% (192 of 296) missing values for preeclampsia and jaundice. The 192 control records also lacked data for other obstetric risk factors. Final record counts by gender and case/control status for obstetric variables were 104 controls, 606 cases, and 558 males, 152 females. Preeclampsia and jaundice prevalence for the sample was 3% and 32%, respectively for the entire sample of 902 records.

Crosstab descriptive frequencies indicated 63% of mothers had a total obstetric risk score of "0", 35% had an obstetric risk score of "1", and 2% had an obstetric risk score of "2 ". Crosstab statistics by ADIR status indicated total obstetric risk scores had fewer than 5 counts for the highest rank score category, so the obstetric risk scale categories were collapsed to reflect a binary responses of "0" or "> 1" (data not shown).

Confounding factors. Infant breathing and sleeping patterns were presumed to be confounders, which may represent underlying congenital heart defects, asthma, allergies, symptoms of psychotherapy medication, comorbid mental health conditions, or ear infections (Hartshorne et al., 2009). These factors were hypothesized to be confounder variables in the relationship to ASD as shown in Figure 2.

Similar or overlapping risk factor and biochemical dysregulation mechanisms were documented for SIDS and ASD (Habek & Kovacevic, 2011; Kinney & Thatch, 2009). Low birth weight, smoke exposure or parental smoking, apnea, previous episodes of interrupted breathing, and hypoxia are SIDS risk factors (American Academy of Pediatrics, 2011; Goldwater, 2011; Van Norstrand & Ackerman, 2010). SIDS-related risk factors appear to more adversely affect male infants than female infants independent of race/ethnicity (Kinney & Thatch, 2009). Burstyn et al. (2011) reported an excess risk of autism among males (*O.R.* 1.0 to 1.6) who were hypoxic at birth for premature and full-term infants diagnosed with fetal hypoxia. Thus infant gender may be an effect modifier as initially proposed in Figure 2.

Analysis was conducted on infant respiratory distress at birth and infant resuscitation at delivery by parental self-report for "Affected Child" and "Unaffected Sib" records. Infant respiratory distress at birth was reported as a binary variable with less than 2.5% missing ($n=23$) values. Resuscitation data were available as binary variable with less than 6.3% missing ($n=27$) values. These variables were compared by gender for all birth types (children of singleton births or multiple birth events). Data was also available for these values for 771 singleton births (233 girls, 538 boys). Child gender did not significantly affect resuscitation required during delivery, but results showed a trend ($p=0.15$) toward higher risk of respiratory distress at delivery for male infants. Multiple birth deliveries were associated with significantly higher risk of both respiratory distress and resuscitation required during delivery ($p = 0.01$). Multiple births reflected 14.5% of records; it was more frequent (13%) in nonfamilial than sib controls (9%).

In summary, the final merged AGRE data sets used ADIR case criteria for autism, and maternal smoke exposure parameters were recoded for temporality (prior, current, or ever) and for direct maternal smoke exposure which was assumed to accurately reflect placental transfer mechanisms proposed in the study framework. Lactation data was recoded to distinguish dedicated and casual lactation from bottle feeding, with the realization that AGRE surveys did not account for other infant beverages or foods, lactation competency or efficacy, or reflect the definition of exclusive breastfeeding established by WHO (WHO, 2001). Quantified maternal prior direct smoking and lactation frequencies showed multimodal distributions. Therefore, the a priori ordinal categorical response scales were used to recode smoke frequency and lactation duration variables. Maternal fish intake data was largely unavailable, and maternal multivitamin use was missing in approximately 40% of the data records. Parental age at first child birth, a priori preconception risk factors were used (including maternal triple screen results that explicitly referred to low maternal folate status), as were proposed obstetric complications (preeclampsia and jaundice delivery). Infant respiratory distress and resuscitation at birth variables were used as proposed confounders for infant breathing variables. These variables were used for cases and controls. Controls reflected familial and nonfamilial child records.

AGRE Sample and Target Populations

Case criteria. The data sample used in this study includes 712 case records for children diagnosed with ASD as defined by ADOS and/or ADIR criteria. The original proposal intended to define positive ASD case diagnosis as ADOS score above a standard

cutoff value and ADIR cutoff value (Norris et al., 2012; Zerbo et al., 2013). However, based on the number of missing ADOS-3 scores, the operational definition of an ASD case child was revised to reflect only positive ADIR scores. This was considered an acceptable revision because ADIR is considered a more strict ASD criteria (Martin & Horriat, 2012). The use of ADOS-1 (three word, spontaneous meaningful phrase speech criteria) ASD diagnosis did not improve data completeness, outcome diagnosis validity, or increase statistical power of the study.

The AGRE target population database was used to compare outcome (ASD) metrics for child records in the Affected Child ($n = 719$) and Unaffected Sib ($n = 190$) databases after exclusions. These outcome populations were shown previously to be statistically valid and accurate based on comparison with the Simons Foundation and Boston Autism Consortium registries (Wall, Dally, Luyster, Jung, & DeLuca 2012). These researchers showed 7 of 93 ADIR survey items could predict ASD case diagnosis with 99% statistical accuracy using an AGRE sample of records from the affected and unaffected databases ($n = 966$).

The ratio of affected boys to girls in the entire AGRE genetic biobank repository is 3.8:1 (Lajonchere, 2010). Within this study, after exclusions and using only ADIR outcome criteria for case definition, the ratio of boys to girls was 2.46:1, and even lower among sibling controls. Twenty records (including 15 males) had ADIR scores of zero, but ADOS-3 (observed behavior) was rated as case positive. Only three child records (two males, one female) showed ADIR and ADOS-3 scores to both be scored as zero. The exclusion criteria applied in this study is similar to that recommended by AGRE

Steering Committee (Geschwind et al., 2001) and directly aligned with the exclusion criteria used by previous AGRE researchers (Anello et al., 2009; Campbell et al., 2009; Cantor et al., 2007; Cheslack et al., 2007; Lee et al., 2008; Martin & Horriat, 2012; Norris et al., 2012; Wallace et al., 2008; Wang et al., 2011).

Controls and unaffected siblings. The small proportion of unaffected sibling records, and even fewer ($n=37$) nonfamilial "true controls" without an affected sibling in this study spurred interest to compare unaffected siblings to "true controls" within the AGRE sample population. Previous AGRE researchers reported sibling patterns which were affected by birth order, gravida, and parental socioeconomic factors (Lee et al., 2008; Martin & Horriat, 2012). Since the purpose of the AGRE cohort is genetic research, the availability of matched controls based on preconception factors has been somewhat limited. Ideally, a case-control study has more than one control per case record. For these reasons, it was worthwhile to compare the response distributions of Unaffected Sibs and "true controls" representing the reference cohort.

Collectively, the AGRE reference group was comprised of 106 complete control records and 190 partial control records as shown in Figure 3. Among the complete control records, 37 children, representing 16 families were identified as nonfamilial true controls, meaning the family code did not contain any siblings having an ADIR score of one within the accessed datasets. Parental ages, gravida, parity, birth type, reference child age, and gender were available for all AGRE reference records. As an aggregate reference group, the subgroup of 296 controls had a male:female ratio of 1.24 (164 males, 132 females). Among nonfamilial true controls, the male:female ratio was 2.70:1. The

average gravida (number of prior pregnancies) was 2.65 among the reference group and 2.69 for the true control group, excluding 5 blank responses. Parity or number of prior births who lived to at least 24 weeks averaged 2.27 for the aggregate reference group and averaged 1.70 for the true control group excluding four blank responses. Among the reference group, 13% of child records were multiple birth pregnancies. Among the 37 true control records, only three children were multiple birth pregnancies.

A subgroup comparison of the proposed nine study variables is shown in Table 8 for the complete records of unaffected siblings and 37 nonfamilial 'true control' records. For vitamin use, 33% of nonfamilial control values and 20% of Unaffecting Sib records were missing or blank values; thus, valid percentages by subgroup of the reference cohort were used. Table 8 data suggests nonfamilial control group was demographically skewed to male offspring, but child age and maternal smoke exposure (ever or prior temporal periods) were similar.

Nonfamilial controls were less likely to be bottle-fed only and had similar or longer lactation duration depending on degree of exclusivity of lactation. Both parents tended to be older in the nonfamilial control subgroup, but with lower preconception and obstetric risk factors. Prevalence rates of infant respiratory distress and resuscitation required at delivery were skewed higher among nonfamilial controls compared to unaffected sibling controls.

Table 8

AGRE Reference Group Statistics

Variable and sample size	Number or units	Unaffected siblings (<i>n</i> = 259)	Nonfamilial controls (<i>n</i> = 37)	Total control group (<i>n</i> = 296)
Multiple births (<i>n</i> =296)	%	13	8	12
Child gender ratio	male: female	1.12	2.70	1.24
Child age (<i>n</i> =296)	mean years	10.1	10.4	10.2
Maternal smoke				
Prior direct (<i>n</i> =161)	%	9.3	10	9.3
Ever exposed (<i>n</i> =150)	%	25	30	25
Prenatal vitamin use (<i>n</i> =227)	%	42	30	40
Bottle fed only (<i>n</i> =146)	%	36	14	30
Breast fed <i>only</i> (<i>n</i> = 146)	%	47	51	46
Lactation duration (<i>n</i> =138)	mean months	5.9	5.0	5.7
Dedicated duration (<i>n</i> =133)	mean months	3.9	7.3	4.2
Maternal age at first birth (<i>n</i> =296)	mean years	31.0	32.8	31.0
Paternal age at first birth (<i>n</i> =293)	mean years	33.3	34.6	33.3

Therefore, the composition of the AGRE reference cohort appeared to be a randomized, diverse subgroup of unaffected siblings which were skewed female, but also included a nonfamilial true control group which were skewed male. The overall prevalence of multiple birth records, lactation durations, and covariates (parental age, preconception risk, obstetric risks) and proposed confounding child traits (sleep traits, asthma, allergies, and respiratory problems) mirrored the ull AGRE sample. These results suggest the control group may be a suitable reference group despite a lower than ideal ratio of the overall number of cases to control records.

Data Collection Summary

For data manipulation and coding, several nonexclusive AGRE data sets were collated and merged, using redundant questions to test internal validity of parental self-reported responses. Parental history files were the primary data source for smoke frequency data due to more complete records, and more specific temporality available in those questionnaires. Maternal fish intake data was very limited and was collected approximately 10 years after AGRE enrollment. Temporality of lactation and degree of dedication of "breast" versus "bottle-fed" practice, and smoking exposures were inspected and recoded using at least two AGRE databases. Affected Child survey responses were the primary data source for lactation initiation and duration, but consistency of responses was compared across AGRE instruments. Detailed univariate analysis of the control group (comprised of familial and nonfamilial controls) was conducted. Results for duration of lactation and maternal smoke frequency were converted to ranked ordinal scores to better reflect the non-parametric data as proposed.

Proposed risk factors of parental age, prepregnancy maternal health (high blood pressure, diabetes, anemia, vitamin B12 deficiency, neural tube defect risk, and albuminuria) and indexed obstetric complications were described as potential covariates. Proposed confounding variables were described via proxy variables of interrupted infant sleep and breathing traits, with consideration by gender and birth type. Multiple birth deliveries were associated with higher resuscitation rates. Respiratory distress and resuscitation showed multicollinearity and was skewed higher among male offspring.

Bivariate analysis for the nine proposed variables hypothesized to be associated with ASD will be discussed in the next section.

Results

Initial Descriptive Bivariate Analysis

In this section, descriptive statistics by ADIR status are presented for all 902 child records. The incomplete child records were not subsequently used to address the research hypotheses, but are illustrated to fully characterize the datasets. Initial analysis include AGRE results for birth type, maternal gravida (total number of pregnancies), and parity (number of pregnancies carried to at least 24 weeks gestation) which provided sample context. Initial descriptive statistics illustrate the lack of maternal fish intake data available, intrapregnancy maternal multivitamin use rates, and relative prevalence of specific types of obstetric and preconception risk factors among the 902 records. After detailing cursory, initial descriptive results, the specific research questions and hypotheses are addressed in the subsequent section on proposed bivariate analysis results.

The initial AGRE demographic cohort traits are shown in Table 9 stratified by both case/control status and gender. Birth type and gravida was not identified a priori as covariates but may impact several study variables such child gender, lactation, smoking behavior, and infant traits. Statistically significant differences indicated in Table 9 are across all birth types (singleton and multiple births). Among controls, singleton births were 87.5% ($n = 259$), and singletons comprised 84.5% of cases ($n = 512$). Among singleton births, gravida, including all non/spontaneous abortions, varied by ADIR status for all birth types ($p = 0.01$). For singleton births, control group mothers ($n = 296$) had

Table 9

Demographic Characteristics of AGRE Cohort by Case Status and Gender

Variable and sample size	N or units	Total (N=902)	Controls (n=296)	Cases (n=606)	Males (n=641)	Females (n=261)
Child age (n=901)	years	9.5	10.2	9.1	9.3	9.8
Maternal smoke						
Prior direct (n=767)	%	38	25*	41*	39	36
Ever exposed (n=756)	%	19	9.3*	21*	19	18
Prior smoke dose (n=681)	cig/day	7.1	7.8	7.1	6.8	8.3
Maternal prenatal vitamin use (n=883)	%	31	40	34	33	26
Maternal weekly fresh fish eaten (n=45)	%					
Lactation practice (n=902)	mo	45	46*	55*	48	38
Dedicated duration (n=402)	mo	4.6	4.2	4.8	4.8	3.9
Any duration (n=763)	%	6.4	5.7*	6.6*	6.5	6.0
Feeding difficulty (n=901)	years	13.7	5.4*	19.8*	14.5	11.9
Maternal mean age (n=902)	years	31.2	31.0	31.3	31.1	31.6
Paternal mean age (n=898)	years	33.6	33.3	33.7	33.4	33.9
Preconception risk						
Diabetes (n=712)	%	1.4	0.7	1.5	1.7	0.8
Hypertension (n=711)	%	8.9	6.7	9.2	9.5	6.5
Anemia (n=710)	%	1.5	1.0	2.0	1.7	1.0
Albuminurea+ (n=710)	%	4.1	4.8	4.0	3.9	4.6
Obstetric risk						
Preeclampsia (n=710)	%	3.0	1.9	3.1	3.6	0.7
Hyperbilirubin (n=710)	%	32.0	31.7	32.0	32.6	29.6
Infant breathing at delivery						
Respiratory distress (n=878)	%	7.0	6.1	7.8	7.8	8.4
Resuscitation (n=874)	%	6.8	8.8	5.8	6.1	5.7

Note. Number of complete records study variables= 733 (556 males, 177 females)

+ Abnormal triple screen comment.

* Significantly different by χ^2 or binomial ($p \leq 0.05$).

2.5 previous pregnancies, 95% CI: [2.4, 2.7] and cases averaged 2.1 gravida, 95% CI [1.9, 2.2]. Parity, differed by gender for all birth types ($p = 0.04$); (data not shown).

Main effect variables. The first three research questions proposed a relationship between maternal smoke exposure, lactation, maternal diet, and fetal gender on the subsequent ASD diagnosis among offspring. Internal and external data prevalence is briefly summarized below.

Table 9 shows maternal ever and prior smoke exposure prevalence. Maternal prior direct maternal smoking prevalence was above 45% for the sample using parental self-report. Tong et al., (2011) reported 33% of younger parents, and 18% older parents reported smoking on birth records in a comparative enrollment period in the US (2004-2008). In this study, there were significant differences ($p < 0.05$) in prevalence rates for ever and prior maternal exposure to smoke, among cases and controls. The AGRE control group reported lower (< 10%) maternal prior smoking behavior regardless of child gender, as shown in Table 8 for the two subgroups (familial and nonfamilial) control records. Nonparametric, independent smoke frequency distributions and medians were analyzed by gender and case/control status using the Mann-Whitney test. Among the 681 responses for quantified exposure to smoke, there were no statistically significant differences between prior or ever maternal smoke dosage distributions or median values of dosage as a function of gender or case/control status (data not shown). While the maternal prior smoke frequency (cigarettes/day) data reflected multimodality, the overall distribution scores skewed higher for cases.

Limited data for the Metals and Mother survey instrument was used to summarize weekly cold-water and fresh water fish intake during pregnancy by trimester. Among 109 matched ID data on maternal fish intake, response rates suggested 66% of mothers ate fish, and 33% of them ate fresh fish while breastfeeding. The prevalence of weekly reported fresh fish intake was higher than that previously reported (6%) for children (Wiest, 2007). But only 45 records were available for quantified fresh fish intake for matched maternal ID codes. Therefore, the sample size for the maternal fish intake variable precluded additional statistical analysis.

Vitamin use rates among the small subsample of maternal fresh fish intake records (30%) appeared to be similar to overall sample vitamin use trends (31%). Maternal vitamin use prevalence rate within the cohort (30%) was similar to other U.S. pregnant women cohort data; 35% (D'Angello et al., 2007; and mothers of a younger birth cohort; 23 to 45% (Sullentrop et al., 2006) which suggested AGRE multivitamin use responses were likely missing at random.

Maternal vitamin use was not significantly different by child gender. Missing maternal vitamin use values were assumed to be similar for multiple birth pregnancies. If maternal intrapregnancy vitamin use was not indicated in older child records, it was assumed not to be used for missing values in subsequent offspring. These assumptions reduced missing vitamin use values in control and case records from 20% to 12% and from 12% to 9%, respectively.

Aggregate lactation practice rates for the AGRE cohort were shown in Table 9. Approximately one-fourth of the AGRE cohort (25% of cases and 27% of control)

reported "bottle fed only" feeding practice responses. "Any" lactation initiation was 54% among cases and 25% of AGRE mothers reportedly used *both* lactation and bottle feeding practices. An aggregate average breastfeeding practice prevalence value of 50% across states within the U.S. was reported for births in 2003-2008 (Ahluwalia, 2012). Maternal self-reported AGRE lactation prevalence was higher than the estimate of Ahluwalia (2012). "Dedicated" lactation practice was reported for 24% cases and 30% of AGRE controls in this study.

Table 9 also showed lactation duration, by case-control status and child gender. Lactation duration was significantly different and higher ($p = 0.05$) among AGRE cases. Mean values within data distributions of dedicated and non-dedicated lactation duration were higher among cases than controls but both variable distributions showed multi-modalities. Dedicated lactation duration did not differ by child gender (see Figure 11). There was suggestive evidence of higher reported incidence of feeding difficulties among male offspring and cases (data not shown but discussed in posthoc analysis section).

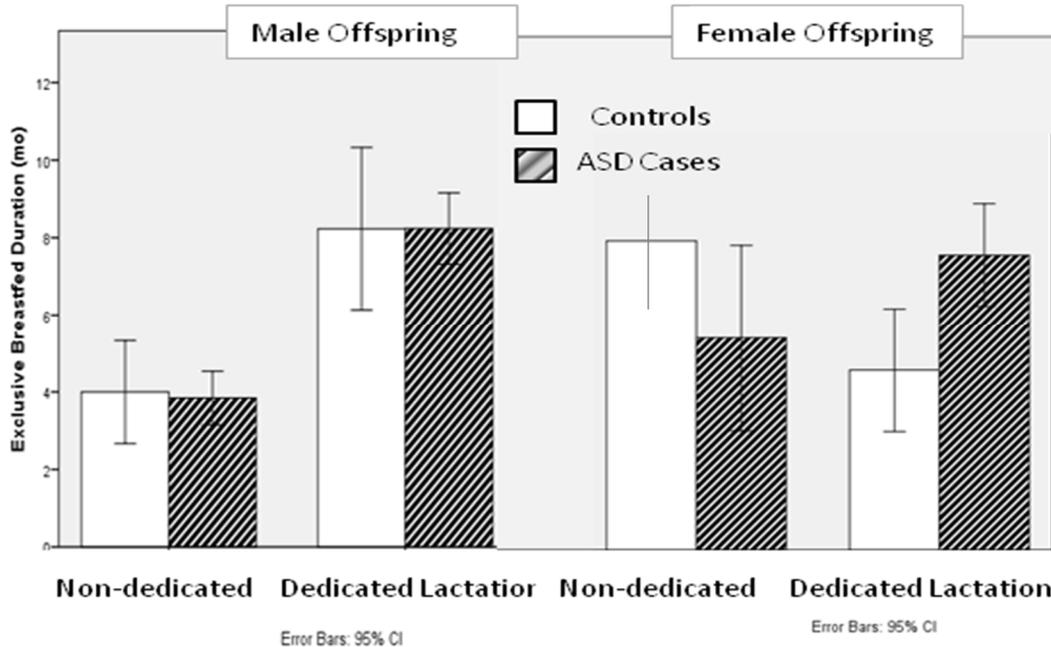


Figure 11. Dedicated lactation duration by gender and case/control status. (n= 402; 70 controls, 332 cases; 85 females and 317 males)

Covariates. Average maternal age was slightly different ($p = 0.11$) by gender. Figure 10 box plot analysis indicated that the distribution was significantly skewed to higher maternal age for female offspring. Average paternal age differed slightly ($p = 0.12$) as shown in Table 9.

Maternal age was recoded per a priori criteria with the assumption that "first born" children were registered as controls or cases within the AGRE registry. Therefore, the data were recoded as maternal age < 36 years at first pregnancy/birth, or greater than or equal to 36 years at first pregnancy/birth. Paternal age was originally defined as a cut-off value of < 20 or \geq 20 years at first pregnancy/birth. However, only three AGRE

records were available for father's age < 20 years. Thus, paternal age data was recoded to binary ages similar to mother age coding.

Maternal preconception health risk factors did not differ significantly by case/control status. There were few cases of maternal preconception diabetes and anemia, but these tended to show gender and ADIR status differences. While the overall maternal preconception diabetes prevalence as shown in Table 9 was less than 1.3% in the sample, preconception diabetes status was reported for 11 male offspring cases, and only 2 female controls. Overall maternal anemia prevalence was also likely low (< 1.5%) as shown in Table 9, but maternal anemia was reported for 11 cases (8 male offspring, 3 female offspring), and only 3 male controls. Similarly, maternal hypertension was slightly higher among cases and males as illustrated in Table 9.

Obstetric risk, (preeclampsia and jaundice) did not differ significantly by case/control status or gender. Overall, jaundice prevalence (32%) was higher than preeclampsia (< 3%).

Prevalence rates for preconception risk factors of maternal diabetes, hypertension, and triple screen test positive results appear similar to that reported in other U.S. autism cohorts (Gregory et al., 2013; Krakowiak et al., 2012; Lawrence et al., 2008). In the AGRE cohort preexisting, diagnosed maternal diabetes was reported for only 13 of 902 records (i.e., 1.3%) in the AGRE cohort as indicated in Table 9. Therefore, it was not possible to compare diabetes prevalence among AGRE cases or controls, or by gender. Hypertension prevalence was estimated to be 8.9% in the AGRE cohort, with slightly higher levels in mothers of cases and male offspring. Krakowiak et al. (2012) reported

preconception hypertension rates of 3.6% in the CHARGE study, whereas Gregory et al. (2013) reported 5 to 7% hypertension in a North Carolina cohort. Across birth type, gender, and among cases, for a given mother, the median difference between preconception and obstetric risk factors was stable and significant ($p = 0.05$). This result may be an artifact of the greater sample power effect for males and cases to detect differences. As a function of increasing risk, preconception and obstetric risk (defined as preeclampsia and jaundice birth delivery) may covary in a positive direction as indicated in previous literature (Duckitt & Harrington, 2005).

Preconception and obstetric statistics, and missing value rate in the AGRE cohort corroborated the results of Wallace et al. (2008) who used similar AGRE data sets to study ADOS case criteria. Preeclampsia was previously defined and validated to include both eclampsia and preeclampsia during pregnancy. Preeclampsia prevalence rates in the AGRE cohort were lower than the 5.2% rate reported by Mann et al. (2010) for a South Carolina cohort of Medicaid recipients. Self-reported hyperbilirubin response rates were used in Table 9 results due to a high rate of missing values in physician-confirmed hyperbilirubin or jaundice. Parental report of jaundice birth rates were higher in the AGRE cohort (mean of 32%) than reported (20 - 21%) for jaundice defined by infant blood bilirubin defined as $> 10\text{mg/dl}$ in California insurance cohort (Croen et al. 2005). The differences in prevalence may be related to differences in the definitional criteria of jaundice or hyperbilirubin (Amin et al. 2011).

Confounding variables. Binary infant respiratory distress and resuscitation required at delivery data at were compared by ADIR status and child gender. Infant males

and cases tended to have higher respiratory distress for both cases and controls (Tables 8 and 9), but there were no significant differences by ADIR status. There was significant correlation between respiratory distress and resuscitation ($p < 0.01$) for the entire sample and by ADIR status. Multiple birth pregnancies were shown to have a three-fold odds risk infant resuscitation of singleton births, regardless of case/control status. Thus, respiratory distress and multiple birth pregnancy data were retained for posthoc analysis of interaction and confounding effects.

Proposed Bivariate Analysis

Independent relationships of pregnancy (main effect) factors and an optimized relationship of pregnancy factors were analyzed per the study design. Proposed bivariate analysis, proportional risk rate results, *chi-squared* results and likelihood ratios among cases and controls, odds ratio and confidence intervals among cases and controls for main predictor variables are described in this section. Using 733 full, complete records, proposed research questions were studied, and main effect relationships to ASD status were optimized to prepare for binary logit regression analysis and to investigate theorized effect modifiers and confounders. Subsequently, preconception covariates, and confounding infant traits to ASD were analyzed for collinearity tests and log regression.

Main effect analysis. The main effect research questions proposed a relationship between prior maternal smoke exposure, lactation, maternal diet and fetal gender on the subsequent ASD diagnosis among offspring. Due to low response rates for fish intake, only the data for maternal vitamin use was used in bivariate analysis to reflect maternal diet factors. Proportional binary response prevalence for maternal prior smoking

behavior, prenatal vitamin use, dedicated lactation, and fetal gender by ADIR status were compared using *Z-test* approximation (Gerstman, 2008). Group differences were significant ($p < 0.05$) for prior maternal smoke exposure, dedicated lactation practice, and gender among cases and controls.

Null hypotheses and alternative hypotheses for main effect relationships are as follows. To increase statistical power directional (one-tail) alternative hypotheses were initially proposed and used in statistical analysis.

Research Question 1: What is the relationship between prior maternal smoke exposure and ASD risk in subsequent offspring within the AGRE cohort?

H_01 : There is no association between prior maternal smoke exposure and ASD.

H_a1 : There is a positive association between prior maternal smoke exposure and offspring ASD in the AGRE cohort.

Research Question 2: What is the relationship between maternal multivitamin intake during or between pregnancy and ASD risk in offspring within the AGRE cohort?

H_02 : There is no association between maternal multivitamin intake and ASD.

H_a2 : There is an inverse association between maternal multivitamin intake and offspring ASD in the AGRE cohort.

Research Question 3: What is the relationship between lactation and offspring ASD risk?

H_03 : There is no association between lactation and offspring ASD risk.

H_a3 : There is an inverse association between lactation and offspring ASD risk.

Research Question 5: How does the exposure-timing relationship of pregnancy variables (maternal smoke exposure, diet, and lactation) to ASD outcome differ by infant gender?

H_{05} : The relationship of maternal diet, prior smoke exposure, and lactation to ASD outcome does not vary by infant gender.

H_{a5} : The relationship of maternal diet, prior smoke exposure, and lactation to ASD outcome does vary by infant gender (i.e., higher in males).

Pearson's *chi-squared* test of likelihood ratio for the probability of rejecting the null hypothesis were analyzed. Likelihood χ^2 ratios for the probability of rejecting the null hypothesis (i.e., there is no associative relationship between the main effect variable and ASD) showed evidence ($p < 0.05$) to reject the null hypotheses that there is no independent association between prior maternal smoking, dedicated lactation, and child gender and ASD risk.

Odds ratio and 95% confidence intervals for one-tailed $\alpha = 0.05$ were calculated for the directional magnitude of the association between main effect variables and case/control status as shown in Table 10. Variable reference events correspond to each set of particular hypotheses statements. Table 10 illustrates significant evidence to reject the null hypotheses that there is no independent association between prior maternal smoking, lactation practice, and child gender and risk of offspring ASD. Specifically, prior maternal smoking, any lactation duration, dedicated lactation practice, and infant male gender were associated significantly ($p < 0.05$) with ASD risk. The directional relationship of self-reported lactation practice and risk of ASD was unexpected.

Table 10

Odds Ratio Confidence Intervals for Main Effect Variables by ADIR Status

Variable	Reference event	Case count	Control count	Odds Ratio	95% CI
Prior maternal smoking % (<i>n</i> =767)	No	273	40	2.48*	[2.30 - 2.57]
Maternal multi-vitamin use % (<i>n</i> =883)	Yes	400	166	1.30	[0.97 - 1.64]
Any lactation % (<i>n</i> =763)	Bottle-fed	347	133	1.42*	[1.00 - 1.97]
Dedicated lactation % (<i>n</i> =763)	No	327	71	1.46*	[1.08 - 2.13]
Child gender (<i>n</i> =902)	Female	479	163	3.08*	[2.28 - 4.16]

Note. CI= Confidence Interval; One-tailed test, $\alpha = 0.05$, * $p \leq 0.05$ for "enter" method

The results showed that among the AGRE sample, mothers who reportedly previously smoked were significantly (2.5 times more likely) to have offspring with subsequent ASD risk than those who did not smoke. Lack of maternal vitamin use tended to be associated with higher (*OR* 1.3) odds of ASD risk in offspring, but the finding was not statistically significant. Children exposed to any lactation duration or dedicated lactation in the sample were 1.4 times more likely to be subsequently diagnosed with ASD. It was theorized *lack of* lactation would be associated with increased ASD risk, so the finding that dedicated lactation was associated with *increased* ASD diagnosis in the sample was unexpected. As expected, male infants in the sample were three times more likely to be diagnosed with ASD than females. Therefore, there is at a minimum, suggestive evidence of significant, directional associations, for magnitudes of odds ratios greater than 1.0 for each of the main effect variables proposed in the AGRE study design.

As theorized, prior direct smoke inhalation and male child gender were independently associated with increased ASD risk. The relationship between maternal vitamin use and ASD risk was non-significant. Contrary to the a priori hypothesis, the binary coded data suggested maternal self-report of any or dedicated lactation practice was associated with increased ASD diagnosis. Temporality of exposures suggest maternal prior smoking was an antecedent ASD risk factor.

To investigate the impact of exposure frequency or duration on ASD risk, the quantified data for prior maternal smoke exposure and dedicated lactation durations were analyzed. The Mantel-Haenszel *chi-squared* statistic was used to analyze the linear test of trends between ordinal levels for prior maternal smoke frequency and dedicated lactation duration (Gerstman 2008, p 468). Table 11 indicates there was no linear trend or independent summary effect by increased strata for prior maternal smoke frequency and ASD risk within the AGRE sample. Expansion of the a priori prior maternal smoke frequency categories resulted in a small cell count (i.e., one control record for prior maternal smoking level of < 5 cigarettes/day) which limited the utility of the Mantel-Haenszel test of trends within smoke frequency groups.

Table 11

<i>Strata Categories of Prior Maternal Smoke Frequency (cigarettes/day)</i>					
Variable	Reference event	Case count	Control count	Odds Ratio	95% CI
Prior maternal smoking	Nonsmoker	315	53		
< 10 cigs/day		81	13	1.05	[0.99 - 1.11]
> 10 cigs/day		<u>186</u>	<u>33</u>	0.95	[0.90 - 1.00]
Group count		582	99		
				<i>Z</i> = -0.06, α = 0.05	

Note. CI= Confidence Interval; One-tailed test, α = 0.05

The results in Table 12 indicate there was no linear trend or independent summary effect by increased strata for exclusive lactation duration and ASD risk within the AGRE sample. Expansion of the a priori categories for values higher than 6 months did not improve the Mantel-Haenszel test of group trends for dedicated lactation duration groups and ASD risk association.

Table 12

Strata Categories for Dedicated Lactation Duration (months)

Variable	Reference event	Case count	Control count	Odds Ratio	95% CI
Dedicated Lactation Duration	Bottle-fed	83	19		
< 2 months		49	10	1.12	[0.99 - 1.11]
2-6 months		79	19	0.95	[0.87 - 1.03]
> 6 months		<u>121</u>	<u>22</u>	1.26	[1.15 - 1.37]
Group count		332	70		

$Z = -0.09, \alpha = 0.05$

Note. CI= Confidence Interval; One-tailed test, $\alpha = 0.05$

Therefore, while proportional differences in prevalence rates for prior maternal smoke exposure and dedicated lactation practice (as binary variables) were significantly different ($p < 0.05$), the quantified exposure frequencies (recalled daily cigarette use and dedicated lactation duration) did not show a linear test of trend with subsequent ASD risk within the AGRE sample.

In summary, bivariate analysis showed significant prevalence rate differences in prior direct maternal smoking, dedicated lactation, and child gender and ASD risk. Hypotheses testing suggested significant evidence (one tailed $p < 0.05$) to reject the null statement (i.e. prevalence differences = zero). Likelihood ratio analysis provided

significant evidence ($p < 0.05$) to reject the null hypothesis that there was no relationship between prior maternal smoking and ASD ($p < 0.01$), dedicated lactation practice and ASD ($p = 0.05$), and child gender and ASD risk ($p < 0.01$). Independent odds ratio analysis results indicated significantly higher risk of ASD for prior maternal smoking exposure, $O.R.= 2.5$, 95% CI [2.30-2.57], male gender, $O.R.= 3.1$, 95% CI [2.28-4.16], and dedicated lactation, $O.R.= 1.4$ 95% CI [1.08 -2.13]. Lack of maternal vitamin use before or between pregnancy was not ($p = 0.45$) associated with higher risk of ASD, $O.R.= 1.3$, 95% CI [0.97- 1.64]. There were no significant, linear trends within reported quantified levels of smoke frequency or lactation duration and ASD status in the sample.

Main Research Question Conclusions

The hypothesized main effect exposure variables proposed to be associated with placental transfer or direct, biological exchange risk factors during pregnancy, and lactation and ASD diagnosis were analyzed using bivariate and odds ratio analysis. Proposed main effect independent variables were studied in their relationship to ASD diagnosis, which was later optimized to derive a logit regression equation for subsequent analysis for other variable effects.

The first research question was “What is the relationship between prior maternal smoke exposure and autism spectrum disorder, defined by ADIR (autism diagnostic interview-revised) score of one?” The results in Tables 9 indicated prior maternal smoke exposure was significantly different ($p < 0.05$) and higher among cases than controls for all birth types. The prior smoke exposure prevalence difference was significantly higher among cases, with significant evidence to reject the null hypothesis that maternal prior

smoke prevalence did not differ. There was also significant evidence to reject the null hypothesis that there was no relationship between prior maternal smoke exposure and ASD risk based on likelihood ratio analysis. Prior maternal smoke behavior was associated with a 2-fold risk of ASD within the sample, $O.R.= 2.5$, 95% CI [2.30- 2.57].

While the sample sizes for quantified prior maternal smoke exposure were small and therefore reduced statistical power, Figure 5 illustrated higher prior smoke frequency among cases (10 to 15 average cigarettes per day of previous smoking by both parents, or mother) than smoke frequency of controls (9 to 10 cigarettes per day). Odds ratio analysis showed more than a 2-fold risk of offspring ASD diagnosis if the mom herself, smoked before pregnancy; and lower odds ratio ($O.R. \sim 1.2$) for contingency table analysis for maternal ever exposed to smoke (data not shown). Therefore, there is sufficient evidence to reject the first null research hypothesis statement regarding no relationship between maternal smoke exposure and ASD risk.

The second research question hypothesis stated there is no association between maternal fish and multivitamin intake during or between pregnancy and ASD outcome within the AGRE cohort. The archived data set for maternal fish intake was largely unavailable for the AGRE sample and was collected 8 to 10 years after AGRE enrollment and completion of other survey instruments. Therefore, maternal fish intake data was not analyzed in bivariate analysis.

Maternal multivitamin use (without temporal qualification) responses were analyzed. Table 9 results indicated there were no significant prevalence rate differences in multivitamin use among mothers of cases and controls. Proportional prevalence rate

analysis confirmed those results. There was insufficient evidence to reject the null hypothesis that there is no association between multivitamin intake and ASD risk. It was postulated that maternal vitamin use would be inversely associated with ASD diagnosis in offspring. Results directionally but not significantly suggest lack of maternal vitamin may be associated with ASD diagnosis. Likelihood ratio analysis suggested a slight probability ($p = 0.09$) that lack of maternal vitamin use was associated with ASD. Odds ratio analysis ($n = 733$ complete records) suggested no significant different odds of ASD for mothers who did not use multivitamins before or during pregnancies.

The third research question proposed an inverse relationship between lactation and ASD risk. The data in Table 9 for all birth types ($n=744$) showed average length of any duration of lactation was higher for cases than controls ($p = 0.05$). Proportional prevalence rate comparisons indicated dedicated lactation practice was significantly ($p = 0.05$) more prevalent among AGRE cases. These results suggested a positive independent association between lactation and ASD risk; which was an unexpected result. Likelihood ratio analysis suggested sufficient evidence ($p < 0.05$) to reject the null hypothesis that there was no association between dedicated lactation and ASD. Odds ratio suggested a 40% higher odds of ASD in offspring reportedly exposed to dedicated lactation, $O.R = 1.46$, 95% CI [1.08 - 2.13].

While the sample sizes for quantified dedicated lactation duration were small and thus reduced statistical power, Table 9 illustrated a higher mean dedicated lactation duration among ASD cases. Figures 9 and 11 also illustrated a trend toward higher maternal self-reported lactation durations among cases. But among the relatively small

number of responses for binary coded dedicated lactation duration ($n= 402$; 70 controls, 85 females) the rank order scores for duration of dedicated lactation did not differ significantly (see Table 12). Therefore, there is sufficient evidence to only suggest a positive association between AGRE self-reported lactation practice in ASD risk. The evidence suggested the relationship between lactation and ASD is not inversely associated, as was initially hypothesized. However, the results were not verified by physician record(s), or adjusted for other liquids, foods, or infant feeding traits.

Factors such as feeding difficulty, and maternal gravida were not identified a priori as potential AGRE study design factors associated with ASD risk. Feeding difficulty was significantly higher among cases and tended to be higher in male infants (see Table 9). Feeding difficulty comments reflected difficulties encountered in breast, bottle feeding, or dual feeding practice. Data on verified lactation practice, competence, adequacy, and the proportional use of lactation and bottle feeding was not available by gender (or case) status. However, feeding difficulties tended to vary by offspring gender as shown in Table 9.

Gender effect on variables was anticipated in the original study design; wherein gender may be a main effect variable or covariate. A one-tailed alternative hypothesis was proposed. Research Question 5: How does the exposure-timing relationship of pregnancy variables (maternal smoke exposure, diet, and lactation) to ASD outcome differ by infant gender.

H_0 5: The relationship of main effect variables to ASD does not vary by gender.

H_a 5: The relationship of main effect variables to ASD does vary by male gender.

Results in Table 9 showed no significant exposure factor prevalence differences by child gender ($N= 902$). ASD prevalence rate differences by gender were significant ($p < 0.01$) as shown in Table 9. Likelihood ratio analysis suggested significant ($p = 0.01$) probability that male children were more likely to be diagnosed with ASD. Odds ratio analysis showed male offspring were three times more likely to be diagnosed with ASD than were female children ($n = 733$).

It was not possible to study the causality of the child gender relationship to ASD risk. However, statistical interaction of AGRE child gender on ASD risk factors was of interest. Stratified analysis of proposed main effect variables (prior maternal smoking and exclusive lactation practice, and maternal vitamin use) by gender showed no statistical interaction. For male children, lower but overlapping odds ratio 95% confidence intervals compared to females were identified for the relationship of prior smoking and ASD.

The results in Table 13 indicated similar risk relationship direction and non-significant differences in magnitudes of odds ratios by gender. The association of prior maternal smoking to offspring ASD diagnosis trended ($p < 0.10$) for both infant males and females, independently. However, the association of lactation practice, maternal vitamin use, multiple birth pregnancies, gravida and respiratory distress to ASD showed no significant gender offspring differential. Therefore, regarding the fifth research question, child gender did not affect the main effect relationships of prior maternal smoking, lactation, lack of maternal vitamin use before or during pregnancy to ASD risk.

Table 13

Odds Ratio Confidence Intervals for Predictor Variables by Child Gender

Predictor variable	Reference	O.R.	95% C.I.	Significance
Prior maternal smoking	Non-smoker			
Male child		1.59	[0.92 - 2.76]	0.10
Female child		2.86	[1.12 - 7.31]	0.03
Lactation practice	Bottle-fed			
Male child		1.50	[0.97 - 2.32]	0.07
Female child		1.42	[0.74 - 2.71]	0.30
Maternal vitamin use	Yes			
Male child		1.49	[0.92 - 2.39]	0.10
Female child		1.53	[0.76 - 3.11]	0.24
Multiple pregnancy birth	No			
Male child		1.27	[0.69 - 2.34]	0.45
Female child		0.68	[0.25 - 1.84]	0.45
Maternal gravida	One child			
Male child		0.88	[0.75 - 1.03]	0.12
Female child		1.04	[0.84 - 1.30]	> 0.5
Respiratory distress at birth	No			
Male child		1.16	[0.37-1.99]	> 0.5
Female child		1.62	[0.09-2.33]	0.24

Note. CI= Confidence Interval for "enter" method; n= 556 male and 177 female children

Logit Regression of Main Effect Variables

The proposed study design involved establishing the hypothesized main effect relationships to ASD risk, with subsequent analysis of proposed preconception covariates and infant trait confounders as shown in Figure 2. Logit regression modeling was used to optimize the predictive relationship of prior maternal smoking, lactation practice, maternal vitamin use, and child gender to ASD risk in the cohort sample for complete records ($n = 733$). The "enter" and "backward likelihood ratio" regression methods were used to minimize suppressor effects of variables (Fields 2005). Table 14 shows the results of hypothesized predictors of ASD.

Table 14

Binary Log Regression Predicting Likelihood of ASD Diagnosis

Predictors of ASD risk	B	S.E.	Exp B	95% CI exp B
Constant	1.27	0.10	3.55	
Prior maternal smoking	0.62	0.21	1.85**	[1.18 - 2.84]
Constant	0.80	0.11	2.21	
Lack of maternal vitamin use	-0.07	0.15	0.94	[0.68 - 1.18]
Constant	1.14	0.13	3.14	
Lactation practice	0.37	0.16	1.47*	[1.05 - 2.08]
Constant	2.17	0.24	8.77	
Gender	-0.64	0.16	2.90**	[2.07 - 3.98]
Constant	0.74	0.08	2.09	
Multiple pregnancy birth	0.13	0.26	1.14	[0.68 - 1.83]
Constant	1.05	0.16	2.85	
Gravida	-0.11	0.05	0.90	[0.81 - 1.04]
Constant	1.31	0.09	3.72	
Respiratory distress at birth	0.41	0.40	1.51	[0.70 - 3.28]

Note. CI = Confidence Interval; n = 733;

* $p \leq 0.05$ ** $p < 0.01$ for "enter" method.

The independent effects of gravida and multiple birth pregnancies were regressed with ADIR. Gravida value (as a range of 1 to 10), and as a cutoff value (< 4) was not a significant risk factor. Multiple birth pregnancy was tested as a binary variable as possible main effect predictor as shown in Table 14. Given the model regressions shown in Table 14, the factor of prior maternal smoking correctly classified 80% of records whereas each other variables predicted only 68% of ASD scores. Further optimization of the regression using multiple predictor variables did not improve correct classification ratio above 80%, but did improve robustness or saturation as shown in Table 15. Field

(2014) reported breastfeeding was associated with maternal gravida. However, the β -coefficient was not improved by more than 10% by the inclusion of gravida or multiple pregnancy births. Therefore, the main effect regression equation shown in Table 15 may reflect the best unbiased estimate of the relationship of the a priori study factors to ASD.

Table 15

Binary Log Regression Optimization of ASD Diagnosis

Predictors of ASD risk	B	S.E.	Exp B	95% CI exp B
Constant	1.79	0.36	5.96	
Gender	-0.61	0.21	1.89**	[1.28 - 2.92]
Prior maternal smoking	0.69	0.22	2.00**	[1.30 - 3.10]
Lactation practice	0.34	0.20	1.47*	[1.08 - 2.08]
Maternal vitamin use	-0.42	0.20	0.96*	[0.52 - 1.04]

Note. N = 733. CI = Confidence Interval.

* $p \leq 0.05$, ** $p < 0.01$ for "enter" method.

The optimized predictive relationship of main effect factors to risk of ASD in offspring was carried forward to test for covariation by preconception health factors of parental age, preconception risk (five health conditions), and obstetric risk (preeclampsia and jaundice). Null hypotheses and alternative hypotheses for proposed covariate relationships are as follows. To increase statistical power, directional, one-tail alternative hypotheses were initially proposed. Research Questions 6-8: How does the exposure-timing relationships between pregnancy exposure-timing variables (maternal smoke exposure, diet, and lactation) and ASD vary by parental age, preexisting maternal health conditions, and obstetric risks?

H_{06} : The relationship of pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD does not vary by parental age.

H_a 6: The relationship of pregnancy exposures (maternal smoke exposure, diet, and lactation) to ASD varies inversely by parental age.

Variable: *Parental age less than 36 years; or > 36 years at first birth.*

H_o 7: The relationship between pregnancy exposures to ASD outcome does not vary by preconception maternal health conditions.

H_a 7: The relationship between pregnancy exposures to ASD outcome varies positively with preconception maternal health factors.

Variable: *A preconception risk (maternal high blood pressure, diabetes, low iron/anemia, vitamin B deficiency/neural tube risk [low folate], albuminuria). (yes/no)*

H_o 8: The relationship between pregnancy exposures to ASD outcome does not vary by obstetric risks within the AGRE cohort.

H_a 8: The relationship between pregnancy exposures to ASD outcome varies positively by obstetric risks within the AGRE cohort.

Variable: *An obstetric complication: (preeclampsia, jaundice delivery) (yes/no).*

Covariate adjustments. Hypothesized covariates for the optimized relationship of preconception and lactation variables and ASD diagnosis were proposed to include parental age, maternal preconception health risk factors, and obstetric risks. Multiple births was not proposed as a covariate; but identified posthoc. The effect analysis of presumed covariates on the relationship of smoking, lactation practice, and gender to ASD will be described in this section.

Parental age regardless of birth types (singleton and multiple births) was not significantly different between cases and controls or by offspring gender. Maternal age showed significant difference by gender among singleton births ($p = 0.03$). Paternal age did not differ by ADIR status, gender or for birth type (singleton or multiple birth pregnancies). Binary responses for mother and father ages at first pregnancy (< 36 years or ≥ 36 years) were used in odds ratio calculations of ASD risk. Odds ratio of ASD risk for mothers 36 years or older was 0.94, 95% CI [0.53 - 1.42]. Odds ratio of ASD risk for fathers 36 years or older was 0.92, 95% CI [0.71-1.33]. Inclusion of mothers age in regression analysis was used in subsequent modeling in keeping with the study construct in Figure 2 for preconception and reproductive health risks.

The AGRE study design presumed singleton birth records, and did not account for maternal gravida or multiple birth pregnancies. But these variables were available in the AGRE data set and analyzed posthoc in attempt to provide context and rationale for the unexpected negative relationship between lactation practice and ASD diagnosis. Maternal gravida and multiple birth pregnancies were identified in the descriptive analysis section of this chapter. Gravida was associated with proposed main effect variables of smoking behavior, lactation, and maternal age. Gravida was further explored in posthoc analysis. Initial regression was not improved using a gravida cutoff (< 4) whereas multiple birth pregnancy data (yes/no) improved the fit of proxy variables for main effect variables with subsequent ASD in offspring. Multiple birth pregnancy data was collinear with maternal cutoff age, and respiratory distress.

Risk of maternal preconception risk tended to skew to male cases, as defined *a priori* for the five criteria listed in Table 9. Odds ratio of ASD risk was only 0.84, 95% CI [0.47-1.49] for binary response (0, > 1) to preconception risk factor score. Odds ratio of ASD for obstetric risk (yes/no tally for either preeclampsia and/or hyperbilirubin birth) was 1.19, 95% CI [0.78-1.82}.

Tests of collinearity between *a priori* and posthoc identified covariates were conducted for the 733 complete records. Prior literature suggested multiple birth pregnancy outcome may be highly collinear with maternal health covariates of preconception risk score, obstetric risk, and/or infant traits of respiratory distress or resuscitation at delivery (Amin et al., 2011; Froehlich-Santino et al., 2014; Gardener & Lyall, 2014; Gardener et al., 2011; Lyall et al., 2014). Using SPSS collinearity diagnostics, maternal age was shown to vary significantly ($p < 0.01$) with multiple birth delivery; 99% of variance was explained by maternal age. Collinearity was not significant for other proposed covariates or confounders (data not shown). One explanation for high collinearity between maternal age and multiple birth pregnancies may be associated with the significant use of fertility treatment (7.1%) in cases compared to controls (1.9%).

To determine whether the variable maternal age or multiple birth delivery should be used due to high collinearity, regression and factor analysis was conducted. Collinear variables were tested for variance inflation factors and factor analysis was used to determine which variables should be retained for regression analysis based on the statistical results (Fields, 2005) and in keeping with the study framework (Figure 2).

Confounders. Infant traits, such as respiratory distress and resuscitation required at delivery were found to be collinear based on variance inflation factors of 1.0. Gender was also found to be collinear with resuscitation at delivery. Infant respiratory distress at delivery was very high among males regardless of case status (see Table 9). Among the 733 complete records, child gender accounted for 97% of the variation in resuscitation response. Odds ratio of subsequent ASD among infants with respiratory distress at delivery was 1.51, 95% CI [0.69 - 3.28]. Odds ratio of respiratory distress among children with obstetric risk (either preeclampsia or jaundice birth) was 2.17, 95% CI [1.23-3.84], $p = 0.03$. Multiple birth pregnancy was also collinear with infant respiratory distress; but the former had lower predictive relationship with ASD risk and lower odds ratio of ASD (OR 1.14) than did infant respiratory distress (OR 1.51). In order to minimize redundant covariate variables, the variable infant respiratory distress was regressed with main effect predictors in log regression.

Infant sleeping and breathing pattern regularity was initially proposed as a confounder. Null hypothesis and alternative hypothesis for the proposed confounding effect are as follows. To increase statistical power a one-tail alternative hypothesis statement was initially proposed. Research Question 4. How is the exposure-timing relationship of pregnancy to ASD confounded by neonatal infant sleeping or breathing traits separately or in combination?

H_04 : The relationship between pregnancy exposures and ASD outcome is not confounded by infant respiratory distress at birth.

H_a4: The relationship between pregnancy exposures and ASD outcome is confounded by infant respiratory distress at birth.

Table 16 shows optimized final log regression of main effect AGRE variables to ASD risk. The table results show infant respiratory distress at birth was a predictive covariate of ASD risk as it was partially correlated with gravida, multiple birth pregnancies, gender, obstetric risk, and maternal age. Independent odds ratio of ASD for infant respiratory distress showed higher mean but overlapping independent odds ratio confidence intervals with gravida and multiple birth pregnancy events as shown in Tables 13 and 14. The use of binary infant respiratory distress response in logit regression analysis improved the predictive equation as shown in the final model of Table 16. However, it is worth noting obstetric risk was significantly associated with infant respiratory distress at birth; but was not significantly related to ASD risk. Infant respiratory distress at birth is likely an antecedent to infant resuscitation at delivery and also may be a conceptual fit with theorized effects of placental transport mechanisms.

Table 16

Binary Log Regression Final Optimization of ASD Diagnosis

Predictors of ASD risk	B	S.E.	Exp B	95% CI exp B
Constant	1.79	0.36	5.96	
Gender	-0.61	0.21	1.89**	[1.28 - 2.92]
Prior maternal smoking	0.69	0.22	2.00**	[1.30 - 3.10]
Lactation	0.34	0.20	1.47*	[1.08 - 2.08]
Maternal vitamin use	-0.42	0.20	0.96*	[0.52 - 1.04]
Constant	1.67	0.31	5.36	
Gender	-0.61	0.20	1.85**	[1.26 - 2.79]
Prior maternal smoking	0.61	0.24	1.84**	[1.13 - 2.95]
Lactation	0.36	0.19	1.44	[0.97 - 2.04]
Maternal vitamin use	-0.07	0.30	0.94	[0.52 - 1.70]
Multiple pregnancy birth	0.16	0.27	1.18	[0.69 - 2.00]
Constant	1.81	0.30	6.12	
Gender	-0.63	0.20	1.89**	[1.25 - 2.77]
Prior maternal smoking	0.59	0.24	1.81**	[1.15 - 2.92]
Lactation	0.37	0.19	1.44*	[1.01 - 2.08]
Respiratory distress at birth	0.32	0.40	1.38	[0.63 - 3.01]

Note. N = 733. CI = Confidence Interval.

* $p \leq 0.05$, ** $p < 0.01$ for "enter" method.

Posthoc analysis. The initial proposal suggested the use of data among singleton births only within the AGRE sample. Due to concerns over sample size, particularly among controls, the inclusion of all birth types (singletons and multiple births) were used for cases and controls. Multiple birth pregnancies reflected 8% of 37 nonfamilial control records, 13% of all 296 control records, and 15% of 606 case records. Birth type as binary response to the question about multiple pregnancy birth was available for all 902 records and used to study covariation. Mean prevalence (15.1%) for multiple pregnancy birth did not differ for the complete 733 records containing values for all nine variables versus the for the full sample (14.8%).

The impact of family size, as estimated by maternal gravida was not proposed in the initial study design. Maternal gravida was available for all 902 records. The mean and median values of gravida (2.45, 2.0 respectively) did not differ for the 733 complete records and full sample. Gravida was shown to be associated with maternal age, smoking behavior, lactation practice and duration, and child gender within the full sample.

Several study variables as a function of gravida were analyzed. The results suggest prior maternal smoke exposure and daily prior smoking frequency (from 5 to 20 cigarettes/day) was reported with increasing frequency for gravida values of 1 to 4 pregnancies. At increasing maternal gravida values (all pregnancy levels), the combined use of breast and bottle feeding decreased, dedicated lactation practice increased, as did lactation duration longer than 6 months. Scott et al. (2008) reported less than 12% of mothers breastfed longer than 12 months; less than 10% of all AGRE mothers breastfed longer than 12 months (data not shown). Gravida analysis showed higher gravida may be related to higher prior maternal smoking duration, and longer lactation duration in the AGRE sample. Gravida as a function of child gender and ASD is shown in Table 17.

Table 17

Maternal Gravida as a Function of Child Gender and Case/Control Status

Study variable response %	----- Gravida by AGRE mother -----						Sample count
	1	2	3	4	5	> 6	
Controls (ADIR = 0)							
Male child record	28.2	25.2	22.7	10.4	9.2	4.3	163
Female child record	22.0	25.8	30.3	12.1	5.3	4.5	132
Cases (ADIR = 1)							
Male child record	26.4	38.6	18.2	11.1	2.5	2.7	477
Female child record	20.2	27.9	29.5	9.3	8.5	4.7	129

Maternal preconception risk as initially defined (preexisting diabetes, hypertension, anemia, low folate/neural tube defect risk, and albuminuria as indicated by abnormal triple screen test result) did not show significant differences among cases and controls, or by gender, as illustrated in Table 9. However, the AGRE cohort population prevalence of preterm labor and pitocin use (a medication used to delay premature labor) had estimated means of 30 and 17% , respectively for the entire sample of 902 records. Pitocin use by mothers of females tended ($p = 0.17$) to be lower (12.5%) than pitocin use by mothers of males, among all records.

Obstetric risk was initially defined and hypothesized to include underlying preconception risks associated with preeclampsia and jaundice birth. Given the initial proposed definition of obstetric risk, these prevalence factors did not differ between cases and controls as evidenced in Table 9. The initial definitional criteria of obstetric risk excluded birth-specific physiological issues such as nuchal cord issues, cesarean section, gestational diabetes, or general edema.

While not originally proposed as component criteria of obstetric risk within this study, there were other pregnancy and delivery factors identified which deserved mention. Specifically, gestational diabetes, general edema, nuchal cord issues, and preterm labor despite the use of pitocin treatment were prevalent within the AGRE database and may inform a more comprehensive perspective of obstetric risks which may mediate the relationship of hypothesized main effect variables with ASD diagnosis in AGRE offspring. However, most of these variables were missing, particularly from control records (192 missing records of 296).

Within the AGRE sample, general edema was frequently (15%) reported. Birth specific nuchal cord issues (a condition in which the umbilical cord is fully wrapped, 360 degrees, around the neck of the fetus at time of delivery) were identified posthoc among the 902 records, as were self-reported responses to questions or comments about preterm labor, pitocin use, and gestational age of specific births. The prevalence of nuchal cord issues differed significantly ($p = 0.05$) among controls (2.9%, SD 0.33) and cases (8.6%, SD 0.34) for all birth records; and for the regression sample records ($n=733$); controls (3.9%, SD 0.33) and cases (9.8%, SD 0.33). Cohort cesarean section delivery averaged 29%, but trended ($p= 0.09$) higher among cases (31%).

Mothers of children of multiple birth pregnancies tended to have higher median obstetric risk index than the obstetric risk index for singleton births. Inclusion of generalized edema and gestational diabetes appeared to significantly increase the median obstetric risk index score for both birth types. The addition of additional obstetric criteria appeared to increase the median obstetric index for children of multiple birth pregnancies more so than the median obstetric index of singletons (data not shown). Inclusion of generalized edema and gestational diabetes appeared to significantly increase the median obstetric risk index score for both birth types. As concluded for the original definition of obstetric risk (preeclampsia and jaundice delivery), preconception factors did appear to covary with the original obstetric risk index. Inclusion of additional obstetric complications did not appear to improve the variable quality or validity.

As discussed previously, multiple birth delivery was highly collinear with maternal age. However, multiple birth delivery was not highly collinear with the initial

preconception score (diabetes, hypertension, anemia, low vitamin B/folate/neural tube risk, albuminuria), obstetric risk (preeclampsia or hyperbilirubin), or infant respiratory distress or resuscitation at birth.

To resolve which covariate factors were redundantly collinear, a broad, posthoc inclusive definition of preconception and/or obstetric risk (including the initial six criteria as well as nuchal cord issues, preterm delivery (< 38 weeks), gestational diabetes, or maternal edema was constructed. Factor regression analysis was conducted with the assumption that total broader preconception and obstetric risk outcome (diabetes, hypertension, anemia, albuminuria, preeclampsia, hyperbilirubin, preterm delivery, nuchal cord issues, maternal edema and/or gestational diabetes) may be associated with multiple birth pregnancies, maternal age, gravida, and/or fertility treatment by case/control status. The results showed statistical significance ($p = 0.05$) for the relationship for the broadly defined obstetric or preconception risk factor to fertility treatment, only for cases; and a statistical trend ($p < 0.10$) for the relationship of the broadly defined obstetric and preconception risk factor to gravida and multiple pregnancy for controls.

Dimensional Factor Analysis

A main effect log regression analysis was proposed to characterize risk of direct maternal smoke exposure and frequency, hypothesized preventive influence prenatal vitamin use, maternal fish intake, and lactation in relation to subsequent offspring ASD risk. The study design presumed the use of singleton birth records; which was not

feasible due to concerns about statistical power for gender analysis. All complete records ($n=733$) for all birth types was used.

As theorized, direct maternal smoke exposure was associated with offspring ASD risk. Unexpectedly, the AGRE data results suggested lactation practice was *positively* associated with offspring ASD risk. AGRE data showed lack of maternal vitamin use and multiple birth pregnancies were also suggestively associated with ASD risk. Odds ratio confidence intervals for these hypothesized main risk factors did not differ by child sex.

Several proposed and covariates identified in posthoc analysis showed collinearity. Maternal age was collinear with multiple birth pregnancy and explained 99% of the variance in the later. Infant resuscitation was highly collinear with antecedent infant respiratory distress at birth. Multiple birth pregnancy was collinear with infant respiratory distress. Infant respiratory distress was also significantly collinear with child gender and gravida.

Therefore, factor analysis was used to minimize redundant effects of proposed covariates and confounders. Maternal age was redundant with multiple birth pregnancy, and infant resuscitation at delivery was redundant with respiratory distress. Collinearity was also tested for preconception, and a priori and ad hoc obstetric factors. Inclusion of infant respiratory distress as a covariate improved risk modeling of ASD. Odds analysis of infant respiratory distress was significantly ($p = 0.03$) associated with overall obstetric risk (preeclampsia and jaundice birth) risks, $OR = 2.17$, 95% CI [1.23-3.84].

Posthoc analysis of covariates was conducted and initial logit regression modeling provided evidence of collinearity among other preconception, pregnancy, and obstetric

risk factors. Factor analysis showed a broad, inclusive (10 point criterion) dimensional factor of preconception diabetes, hypertension, anemia, albuminuria, gestational diabetes, maternal edema, preeclampsia, hyperbilirubin, preterm delivery, and nuchal cord issues was significantly ($p < 0.01$) associated with fertility treatment use among parents of offspring cases.

Odds ratio analysis showed infant respiratory distress had higher odds ratio (1.51) than multiple pregnancy births (1.14), or gravida (0.09) in the independent relationship to ASD risk as shown in Table 14. Infant respiratory distress fits the conceptual theory in Figure 2 and was retained in the final regression which improved ASD odds ratio values of main effect variables.

Based on the initial main effect research questions, the study results suggested prior maternal cigarette smoking as well as indirect smoke exposure was associated with increased ASD risk based on odds ratio analysis (OR 2.5 and 1.2, respectively). There was a significant and positive relationship between daily maternal smoke exposure and higher ASD risk in the AGRE sample. Directionally, lack of maternal vitamin use was suggestively positively associated with ASD risk with moderately higher odds ratio ($OR=$ 0.96, 95% CI [0.52-1.04]). The hypothesized *positive* relationship between AGRE lactation practice variables and lower ASD risk was *not* observed in the AGRE data. Maternal self-report of lactation practice (lactation without bottle use) was associated with increased risk of ASD diagnosis. Lactation practice was associated with higher subsequent ASD diagnosis, $OR=$ 1.47, 95% [1.05 - 2.08] as an independent predictor and

was a significant ($p = 0.05$) predictor in regression analysis. Temporality of maternal prior smoking as a risk factor was antecedent to lactation practice.

Male infants had significantly higher respiratory distress and higher prevalence of resuscitation at delivery than female infants. Respiratory distress was higher among males, regardless of case status. Multiple birth pregnancies were shown to have a three fold odds risk infant resuscitation of singleton births, regardless of case/control status. For these reasons, birth type (singleton or multiple) was used in initial regression analysis to predict likelihood of ASD.

Table 16 illustrated the optimized, hypothesized proxy placental transfer variables which were associated with ASD for the AGRE data set ($n = 733$). The risk factors included in the optimized regression include the hypothesized proxy variables for placental transfer mechanism (prior maternal smoking, maternal vitamin use), and possible placental or nutritional transfer of lactation practice, and the well-known gender differential of ASD risk. The use of maternal vitamins before or during pregnancy were not a significant predictive factor to ASD risk in this study. Infant respiratory distress was theorized to be a confounder, but results indicated it was a covariate and a temporal antecedent to lactation practice. Likewise, prior maternal smoking would be a temporal antecedent risk factor to subsequent infant respiratory distress at birth. Inclusion of infant respiratory distress as a risk variable in the model improved statistical significance of lactation practice in the predictive equation of subsequent offspring ASD (see Table 16).

Summary of Results

The goal of the proposed retrospective, case-control design was to articulate an exposure-timing relationship for ASD risk. Minimal uncertainty bias, measurement and recall bias with regard to preconception health behaviors, pregnancy and lactation data to investigate gender differentiated ASD risk was a secondary goal. The author proposed risks hypothesized to be associated with placental transport mechanisms, with adjustment for unique obstetric risks and infant traits which may confound the relationship of dominant risk factors of ASD, and the ASD gender differential.

After conducting cursory descriptive analysis, independent odds ratios associated with pregnancy factors and ASD were calculated to address the main effect research questions. The results showed there was sufficient evidence to reject the null hypotheses for the association of prior maternal smoking and risk of ASD. There was also sufficient statistical evidence ($p < 0.05$) to reject the null hypotheses for the association of lactation and risk of ASD: And to reject the null hypotheses for the association of infant gender to risk of ASD in the AGRE sample. The AGRE data analysis failed to reject the null hypotheses that there was no association between maternal intrapregnancy multivitamin intake and ASD risk.

Predictive regression for complete records for all birth types ($n=733$) showed significant odds ratio for prior maternal smoking, $OR= 2.00$, 95% [1.30 - 3.10], male infant gender, $OR= 1.89$, 95% [1.28 - 2.92], and lactation, $OR= 1.47$, 95% [1.08 - 2.08] associated with ASD risk. Proposed covariates of maternal age, preconception risk factors, and obstetric risk did not improve the predictive regression equation.

Proposed and post-hoc identified covariates exhibited collinearity and infant respiratory distress was a covariate, rather than a confounder in the optimized predictive relationship of prior smoking, lactation and infant gender and ASD risk. After conducting factor analysis to resolve variable collinearity, infant respiratory distress data was identified as a key covariate and improved the predictive relationship of ASD risk factors. The clinical and temporal significance of the statistically significant predictive regression equation will be described in the next chapter as will social change and research implications.

Chapter 5: Discussion, Conclusions, and Recommendations

Introduction

Study Purpose

The goal of the retrospective case-control study was to describe the hierarchical and temporal relationship and odds risk between pregnancy related risk factors associated with plausible placental transfer mechanisms to ASD outcome and the ASD gender differential. The use of behavioral survey data collected as ancillary information for a AGRE was anticipated to provide data of minimal uncertainty, measurement, and recall bias. An autism registry sampling frame was used in attempt to gain adequate response rate for female children with ASD in order to study the elusive but persistent ASD gender risk differential.

The purpose of this study was to describe the relationship between direct maternal diet and tobacco smoke exposures during pregnancy and lactation, distal preconception risk exposures, confounding infant breathing traits and the risk of ASD, and gender differential of ASD per the initially proposed study framework in Figure 2. It was hypothesized that placental transport risk factors of prior maternal smoking, lack of maternal vitamin use, lack of breastfeeding, and male gender were associated with higher odds offspring ASD risk. There is little prior literature on the effect of maternal diet and lactation practice on ASD risk. There is also little published information for temporality data on preconception and pregnancy risk factors associated with the ASD gender differential, which has been shown to be consistently observed.

Archived AGRE data discrepancies, variable definitions, data validation, coding, and identification of 733 complete records among 902 raw records were described in Chapter 4. This data set was used to test the research questions associated with plausible placental transfer mechanisms as main effect variables in the relationship to ASD and gender differential of ASD. The initial intent was to also prepare the AGRE dataset for logit regression analysis for the assumption of singleton birth children. However, due to concerns over statistical power, all birth types were used in bivariate and regression analysis ($n = 733$). Maternal gravida, or number of pregnancies, was not considered a priori but covaried positively with maternal age, prior smoking behavior and frequency, lactation practice, and dedicated lactation practice and duration. Factor analysis identified infant respiratory distress as a key covariate and its inclusion in the predictive regression modeling improved the predictive risk equation for ASD.

Key Learnings

In the first research question, the null hypothesis was rejected, and the alternative hypothesis was accepted. There was a positive association between prior direct maternal smoke (binary) exposure and offspring ASD outcome. Independent odds ratio of ASD risk was estimated to be 2.48, 95% CI [2.39-2.57] among mothers who previously smoked compared to nonsmokers, with lower odds ratio (~ 1.2) for mother previously or ever indirectly exposed to smoke.

For the second research question, maternal diet data for maternal fish intake was not available for analysis. Maternal multivitamin intake had an adequate response rate to be concluded in independent odds ratio analysis, but there was statistically insufficient

evidence to reject the null hypothesis. Odds ratio analysis was not statistically significant; odds of ASD diagnosis for mothers who did not report maternal vitamin use was 0.96, 95% CI [0.52- 1.04]. There was a suggestive trend ($p < 0.10$) for an inverse association between multivitamin intake (before, during, and/or between pregnancies) and offspring ASD risk in the predictive modeling equation.

There was sufficient evidence to reject the third null hypothesis (i.e., there was no association between lactation and ASD risk). However, the stated alternative hypothesis of an inverse association between lactation and ASD risk was also rejected. There was a significant association between recalled, self-reported lactation, and ASD risk in the AGRE sample. However, dedicated lactation practice was positively to ASD risk. The odds risk of ASD was estimated to be 1.47, 95% CI [1.08 - 2.08] for AGRE mothers of children attempting dedicated lactation compared to those who were only bottle-fed.

The fourth research null hypothesis stated the optimized relationship among placental transport proxy variables (maternal direct smoking, lactation, and multivitamin use) was not confounded by infant breathing traits. The data analysis showed infant respiratory distress at birth was a predictive covariate, rather than a confounding variable in the relationship of the main effect variables and offspring ASD risk. Therefore, there was insufficient evidence to reject the null hypothesis. Odds ratio of ASD diagnosis was estimated to be 1.51, 95% CI [0.70 - 3.28] for AGRE children whose parents reported infant respiratory distress at birth. Inclusion of respiratory distress as a covariate improved the statistical significance of hypothesized, predictive main effect of prior

maternal smoking, lactation, and infant gender to ASD risk. Infant respiratory distress was also collinear with maternal age and binary obstetric risk data.

The fifth research question addressed whether child gender mediated the effect of plausible placental transport mechanism proxy variables and offspring ASD diagnosis in the AGRE cohort. There was evidence to reject the null hypothesis (i.e., the relationship of maternal diet, prior smoke exposure, and lactation to ASD outcome does not vary by infant gender). The relationship of prior maternal smoke exposure, lactation, and maternal multivitamin use did not vary significantly by child gender as shown in Table 13. Odds ratio confidence intervals overlapped but were directionally similar with regard to prior maternal smoke exposure, lactation practice, maternal multivitamin use before and during as well as for gravida and multiple pregnancy births. However, infant gender was a predictive main effect variable: Odds ratio of ASD was estimated to be 2.90, 95% CI [2.07 - 3.98] among male offspring in the AGRE sample.

The sixth research question proposed the relationship of proxy variables of placental transport mechanism (maternal smoking, lactation, multivitamin use) varied inversely by increased parental age. Odds ratio for ASD risk was not significant for higher maternal and paternal age at first pregnancy/birth (< 36 years and \geq 36 years). Odds ratio of ASD by parental age was 0.94, 95% CI [0.53-1.42] and 0.92, 95% CI [0.71-1.33], respectively for mothers or fathers 36 years or older. Maternal age was collinear with respiratory distress and multiple pregnancy births. Inclusion of maternal age as a binary variable did not improve the statistical regression equation. Therefore, there was insufficient evidence to reject the null hypothesis; parental age did not affect the main

effect relationship between maternal smoking, dedicated lactation, maternal multivitamin use, and child gender to ASD risk in the AGRE sample.

The seventh research question null hypothesis stated the relationship between pregnancy exposures did not vary preconception risk using five criteria. The results showed preconception risk (yes/no) did not statistically impact the significant relationship between prior maternal smoking, dedicated lactation, maternal vitamin use, and fetal gender and ASD risk in the AGRE cohort. Odds ratio of ASD was 0.84, 95% CI [0.47-1.49] for preconception risk. Therefore, there was insufficient evidence to reject the null hypothesis. In addition, the predictive relationship of pregnancy, main effect factors and ASD did not vary by preconception risk status.

The eighth null hypothesis stated the relationship between pregnancy exposures to ASD outcome does not vary by obstetric risk (defined by preeclampsia and/or hyperbilirubin delivery). Independent odds ratio of ASD in the AGRE sample for mothers who reported an obstetric risk was 1.19; 95% CI [0.78-1.82]. Obstetric risk was strongly associated with infant respiratory distress at delivery OR= 2.17, 95% CI [1.23-3.84], $p = 0.03$ but was not a significant covariate or confounder on the main effect relationship. Obstetric risk also was shown not be to significantly associated with ASD. Thus, there was insufficient evidence to reject the null hypothesis for obstetric risk. Inclusion of obstetric risk did not improve predictive models.

In conclusion, the log regression predictive of ASD likelihood within the sample can be expressed as the following based on modeling shown in Table 16 for the sample:

$$Y(\text{ASD diagnosis}) = \beta 2.0(\text{prior maternal smoking}) + 1.4(\text{dedicated lactation}) + 1.9(\text{infant male gender}) + 1.0(\text{lack of maternal vitamin use})$$

The above equation did not adjust for the factor of infant respiratory distress at birth (yes/no). The inclusion of posthoc identified covariate of infant respiratory distress in the regression model shown in Table 16 resulted in the following predictive equation:

$$Y(\text{ASD diagnosis}) = \beta 1.8(\text{prior maternal smoking}) + 1.4(\text{dedicated lactation}) + 1.9(\text{infant male gender}) + 1.3(\text{respiratory distress at birth})$$

Interpretation of the Findings

External validity. The bivariate analysis showed prior direct maternal smoke exposure prevalence was representative of other similar populations and higher among mothers of cases than controls. Measurement or recall bias of smoking behavior has been theorized to be strongly confounded by maternal education level, other socioeconomic variables, and mode of data collection (i.e., birth certificates versus medical records) in several studies (Dietz et al., 2011; Kalkbrenner et al., 2012; Lee et al., 2012; Burstyn et al., 2012; Vinikoor et al., 2010; Zhang et al., 2010).

AGRE data for parental reported prior direct smoke inhalation by mothers showed mothers of ASD cases had significantly higher maternal prior smoke prevalence rates than mothers of controls. Maternal smoking during pregnancy is a common risk factor for fetal hypoxia, which may be associated with placental insufficiency based on ultrasound verification at 10 to 20 weeks gestation (Habek & Kovacevic, 2011). Among mothers

who smoked more than 10 to 20 cigarettes per day, risks of fetal hypoxia was much higher than nonsmoking mothers. In this study, no significant differences were reported among parental-reported prior maternal smoking frequency (cigarettes per day) among mothers of cases and controls. This result may be related to the observation that smoking response rates and prevalence reported varied by AGRE survey instrument; therefore, response distributions were broadly nonparametric as shown in Figure 5. Additionally, the use of an open-text AGRE questionnaire response scale may have led to clustering of data, based on fractions of cigarette packs rather than an assumed continuous scale.

Odds ratio of ASD diagnosis was estimated to be 2.48, 95% CI [2.30-2.57] among mothers who previously smoked compared to nonsmokers, with lower odds ratio (~ 1.2) for mothers previously or ever indirectly exposed to smoke. The point estimate of odds ratio in this study was higher than odds ratio reported by Kalkbrenner et al. (2012) and Mann et al. (2010) for U.S. populations but similar to those reported by Grazuleiciene et al. (2009) for smoking during pregnancy. The higher odds ratio observed in this study may be due to the use of three sources of parental smoking AGRE data or temporality of smoking (before pregnancy). Overall, data illustrated a significant and positive relationship between direct maternal smoke exposure and ASD case diagnosis in offspring. Due to multimodality of prior maternal smoke frequencies in the sample, there was no significant trend in smoke frequency (cigarettes smoked per day) to ASD risk despite access to three AGRE questionnaire data sources.

Key findings from the exploration of maternal diet factors of intrapregnancy vitamin use and fish intake during pregnancy and lactation showed lack of maternal

vitamin use tended to be associated with increased odds risk of ASD in offspring, but the finding was not statistically significant. Odds ratio analysis showed ASD diagnosis given lack of maternal vitamin use was not significant, $OR= 0.94$, 95% CI [0.68 - 1.18]). Odds ratio obtained in this study is similar to odds, $OR= 0.6$, 95% CI [0.4 - 0.9] reported by Schmidt et al. (2011) for first trimester maternal vitamin use ($n = 429$) in a California study. Periconception timing and prenatal vitamin and folic acid fortification was shown to be significant in the first trimester (Lyall et al., 2014; Steenweg-de Graaff et al., 2014).

There was insufficient response rate available for statistical analysis of maternal fish intake. Thus, there was a lack of evidence to analyze or conclude the direction or magnitude of the relationship of maternal weekly fresh fish intake to subsequent ASD risk in offspring defined by ADIR score. The survey instrument data for fresh fish intake was also collected more than 8 years after initial AGRE family enrollment and used a different AGRE sample. There was concern dissimilar timing of data collection for other variables, and fish intake may contribute history or maturation bias risks. Thus, maternal fish intake bivariate results were not available.

The results for the hypothesized inverse association of lactation practice and duration with ASD suggested the relationship was not accepted. The alternative hypothesis predicted a positive association between any or dedicated lactation practice and ASD. While complimentary breast and bottle practice was reported in many AGRE cases and control records, there was good internal data agreement for bottle-fed only prevalence and dedicated lactation practice as coded from the AGRE questionnaire. The independent odds risk of ASD was estimated to be 1.47, 95% CI [1.05 - 2.08] for AGRE

children whose mothers reported dedicated lactation compared to those who were exposed to bottle feeding. Lucas (2011) and Field (2014) concluded atypical infant latching, suck, swallow, and insatiable feeding among at-risk and ASD cases based on retrospective study designs. Parental psychopathology may confound the social, bonding, and nutritional benefits typically associated with breastfeeding practice (Field, 2014). It seems plausible maternal nutritional status, abnormal fetal development, or breathing issues may affect ability of infants subsequently diagnosed with ASD to perform nutritive breastfeeding capacity.

Lactation duration greater than 6 months tended to be associated with higher ASD risk in the AGRE sample. Again, these results were not consistent with the hypothesized relationship. Li et al. (2014) reported that infants ($n = 1,281$) exclusively breastfed by WHO definition beyond 6 months had lower odds of sinus, ear, nose, and throat infections at age 6, but breastfeeding practice had no effect on upper respiratory or lung infections. Infant records were not adjusted for respiratory distress at birth in that study.

Shamberger (2011) reported breastfeeding practice was associated with higher ASD risk among children of WIC families--a federal grant program for supplemental foods, health care referrals, and nutrition education for low-income pregnant, breastfeeding, and nonbreastfeeding postpartum women, and to infants and children up to age 5 (FNS, 2012). In the Shamberger study, infants who were exclusively breastfed tended to have diets with lower thiamine, riboflavin, and vitamin D than U.S. minimum daily requirements for these nutrients. In this AGRE study, nutritional status of mothers was designed to be approximated by maternal vitamin use and weekly maternal fish

intake. However, maternal fish intake data was largely unavailable. Multivitamin use results were not significantly related to ASD risk, and the cross-tabulation analysis of maternal multivitamin use and dedicated lactation practice was not significant. The AGRE study results were not comprehensive enough in scope to address whether lactation competence, feeding difficulties, and/or maternal nutritional status are associated with multivitamin intake and/or offspring nutritional status. In addition, external validity of the effect and relationship of lactation to subsequent ASD risks is difficult to access due to a lack of published literature on the duration, degree of dedication, and subsequent offspring ASD risk.

Access to the AGRE phenotypic and behavioral trait datasets provided an opportunity to review several preconception risk factors associated with ASD. Because the nature of the AGRE repository is genetic research, the control group included unaffected siblings, but also contained nonfamilial true control records. The composition of the AGRE reference cohort appeared to be a randomized, diverse subgroup of unaffected siblings which were skewed female, but also included a nonfamilial true control group which were skewed male. The overall prevalence of multiple birth records, lactation durations and covariates (parental age, preconception risk, obstetric risks) and proposed confounding child traits (sleep traits, asthma, allergies, and respiratory problems) mirrored the full AGRE cohort. The results suggest the control group was a suitable reference group despite a lower than ideal ratio of the overall number of matching controls to case child records.

Results for maternal direct smoke exposure replicated previous published prevalence data for U.S. sample populations (PRAMS, 2004). In this study parents more frequently admitted to higher prevalence and daily frequency of smoking in prior periods (prior to child enrollment in AGRE, including teen, young adult and preconception period) relative to current period (at AGRE enrollment when offspring age averaged 8 to 9 years). The results were similar to other parents in the US for birth reports for 2002 to 2008 (Tong et al., 2011). In this study, temporality of smoking, and access to three separately collected data sources may have minimized data bias.

In this study, internal data validity was verified using multiple AGRE survey instruments (all administered during initial family enrollment). Odds ratio analysis indicated odds of ASD diagnosis was 2.5 for mothers who previously smoked, and *OR* was 1.2 for mothers previously or ever exposed to secondary smoke. The effect of smoking during pregnancy showed a summary effect or average odds ratio of 1.2 for four previous studies (Gregory et al., 2013; Kalkbrenner et al., 2012; Grazuleiciene et al., 2009) as shown in Table 3. Tran et al. (2013) also reported an odds ratio of 1.2, 95% CI [1.0 - 1.5] maternal smoking throughout pregnancy and autism; and the increased odds persisted after controlling for maternal age, socioeconomic and psychiatric status, and infant's weight for gestational age. The AGRE data results suggested maternal gravida was positively associated with prior direct maternal smoking behavior and daily smoke frequency. But maternal smoke frequency and gravida tended to be higher for female offspring, and among female controls compared to male cases or male control offspring.

McDonald et al. (2006) reported maternal smoking elevated umbilical cord levels of adrenocorticotropin hormone among 104 infants delivered by elective caesarean section. The authors speculated cigarette smoking may be associated with hypoxia-related events as a result of hypothalamic-pituitary-adrenal axis involvement in "fetal programming" responses resulting in increased carboxyhaemoglobin, reduced placental oxygenation and uterine vessel vasoconstriction. Pedersen et al. (2013) reported presence of DNA placental cord blood methylation adducts was associated with maternal direct or secondhand smoke exposure and associated with intrauterine growth restriction.

Conceptual framework. The temporal conceptual framework for this study was adapted from Burstyn et al. (2011). The researchers concluded fetal hypoxia was an effect variable in the relationship to ASD after adjustment for socioeconomic status, birth year, and fetal gender.

Plausible explanatory hypotheses for hypoxia-ASD relationship described in the Burstyn et al. (2011) study included prenatal dopamine (serotonin inhibitor) exposure, maladaptive stress responses and/or gender-specific placental physiology. Stewart and Klar (2013) reported that double branching of bronchi in the lower lung airways (as opposed to typical, single branching) was a unique feature among children with ASD patients referred to an Arizona childrens' hospital between 2009 and 2011. A total of 459 children were tested; all 49 children with ASD were also shown to exhibit doubled-branching bronchi via photographic inspection. Habek and Kovacevic (2011) reported children of mothers who smoked had a high rate of fetal hypoxia verified by utero ultrasound, and bronchoconstrictive syndrome at birth.

Infant breathing or sleeping patterns were not expected to be main effect variables in this study as they may reflect congenital heart defects, asthma, or allergies. Robust methodology and survey instruments for characterizing infant sleeping and sleep apnea traits have not been standardized (Mahoney & Caterino, 2011; Young et al., 2009). However, the AGRE datasets contained binary responses to infant respiratory distress at delivery, and resuscitation (yes/no) at delivery for each of 902 records in the sample. These data were used to test the hypothesized confounding effect of infant breathing traits on the relationship of maternal health to ASD risk. Infant respiratory distress and resuscitation at delivery, by gender and birth type was studied. More than 85% of child records were for singleton births, with 14.5% reflecting multiple births. The rate of multiple births did not differ significantly for the 733 records used in predictive logit regression analysis of ASD risk.

In this study, odds of infant respiratory distress was higher among male infants, regardless of case status. There was significant correlation between antecedent infant respiratory distress and resuscitation ($p < 0.01$) for the entire sample and by ASD status, which is logical. Respiratory distress was shown to be collinear with gravida, maternal age, multiple birth pregnancies, and obstetric risks. Factor analysis showed infant respiratory distress to be a critical covariate in the relationship of maternal smoking, lactation, and gender to offspring ASD. Inclusion of the binary response to infant respiratory distress (yes/no) improved predictive regression modeling more so than inclusion of maternal age, gravida, or multiple birth pregnancy. Froehlich-Santino et al. (2014) reported respiratory distress, $OR = 2.29$, 95% CI [1.12-4.67] and other markers of

hypoxia, $OR= 1.99$, 95% CI [1.04-3.80] were associated with increased ASD risk in males in a California cohort of 194 twin pairs studied at Stanford University and diagnosed using aligned Standard University and AGRE definition of ASD.

Temporality. AGRE lactation results suggested cases tended to have higher lactation duration even when *both* breast and bottle feeding practice was used. Parental response to dedicated lactation practice was reported to be more prevalent among cases, and male offspring, despite higher reported feeding difficulties among male offspring and ASD cases in the AGRE sample. The result that dedicated lactation was associated with increased risk of ASD was surprising but prevalence ratios mirrored other studies. Prior maternal smoking would be an antecedent factor to subsequent respiratory distress at birth, and lactation practice. In this study, odds ratio analysis for dedicated lactation, given prior maternal smoking behavior was suggestive ($p = 0.10$) but insignificant, $OR= 1.33$, 95% CI [0.94-1.9]. Therefore, there was no significant correlation between maternal prior smoking and lactation practice among mothers. Alternatively, it is possible and consistent with the proposed theoretical framework that maternal smoking affected placental sufficiency, brain development, and fetal hypoxia (Habek & Kovacevic, 2011). These factors in turn, may affect respiratory distress at delivery, and in turn, affect subsequent nutritive breastfeeding capacity.

Field (2014) and Lucas (2011) reported children with development delays and ASD had more difficulty with the muscle and breathing coordination required for nutritive breastfeeding. Lucas (2011) reported that bottle-feeding may be less strenuous for newborns. Schultz et al. (2006) reported risk of ASD was higher among infants who

were not breastfed, or fed infant formula without DHA fortification. Further, in an ecological study of mother's enrolled in the Supplemental Nutritional Assistance Program (SNAP; previously named Women's Infant and Children or WIC) showed exclusive breastfeeding was associated with lower thiamin, riboflavin and Vitamin D status among infants (Shamberger, 2011). Periconception folate levels have been shown to be significant predictors of ASD risk in offspring (Braun et al., 2014b; Lyall et al., 2014; Neggers, 2014; Steenweg-de Graaff et al., 2014). Mothers who smoke (as evidenced by serum cotinine levels) reportedly have reduced blood folate levels (Prasodjo et al., 2014).

In the AGRE sample, increased maternal gravida was shown to be associated with dedicated lactation practice, and dedicated lactation duration longer than six months. Feeding difficulty was also higher among male infants, which tended to be skewed to ASD case status. Giglia et al. (2006) reported that for a 12 month longitudinal study in Perth, Australia, women who smoked during pregnancy had lower prevalence and shorter duration of breastfeeding (average of 28 weeks versus 11 weeks) than nonsmoking mothers even after adjustment for maternal age, education, income, father's smoking status, breastfeeding intention, birth weight or mother's country of origin. In this study, the odds ratio point estimate for dedicated lactation, given prior smoking behavior was suggestively ($p = 0.10$) positive, but insignificant $OR = 1.33$, 95% CI [0.94-1.9].

A possible explanation in the AGRE study sample for the association of dedicated lactation and ASD risk may be that dedicated lactation practice and duration was also associated with multiple birth pregnancies and higher maternal gravida. There was suggestive evidence that dedicated lactation practice may be related to lack of optimal

maternal health and/or feeding difficulties. Due to multimodality of AGRE dedicated lactation duration data, there was no significant linear trend in lactation duration to subsequent ASD risk. Multiple birth pregnancy events and gravida was shown to be positively associated with dedicated lactation practice. Due to archival data source limitations, lactation duration data were not adjusted for maternal health status, lactation verification by physician records, lactation capacity or efficacy.

Schultz et al. (2006) reported that mothers who recalled using infant formula without docosahexanoic acid and arachidonic (DHA) had more than 4-times the risk of ASD; and mothers who did *not* breastfeed for the first six months had more than 2-fold the risk of offspring ASD diagnosis in an internet convenience sampling of ASD cases ($n = 861$). Dodds et al. (2011) reported $OR = 1.2$, 95% [1.0 -1.4] of offspring ASD among mothers who initiated breastfeeding in Nova Scotia hospitals ($n = 924$). The inverse relationship and OR magnitude between lactation and ASD in the AGRE study are similar to results of Dodds et al., (2011). Therefore, the unexpected positive association of lactation and ASD may be coincidental or affected by infant respiratory distress, nutritive suck, maternal health status, gravida, or parity.

Posthoc results. Covariate analysis was studied for the proposed effect modification of maternal preconception health, parental age, and obstetric complications associated with the pregnancy or delivery. Gravida was found to be a covariate for several study variables including maternal age, child gender, lactation duration, and smoking behavior. Paternal age did not differ by case/control status or gender, but maternal age was significantly higher among females, and among controls. Maternal

gravida also tended to be higher among female controls compared to male controls or male cases. However, these covariate were collinear with respiratory distress.

Maternal preconception health factors of diabetes, hypertension, anemia, low folate/neural tube risk/low vitamin B, and albuminuria by abnormal triple screen test did not differ among cases or controls. There was a possible suggestive trend for increased preexisting diabetes, anemia, gestational diabetes, in male offspring but the sample counts were very low (less than 1.5% of 902 records in the population). Prevalence rates for preconception risk factors were similar to other researcher findings and geographic sample groups. It was of interest that despite diligent maternal triple screen testing prevalence in the AGRE sample, less than one third of abnormal triple screen results were reportedly followed up with amniocentesis testing based on parental information.

Obstetric risk of preeclampsia and hyperbilirubin also did not differ by case/control status or offspring gender. For each maternal-child record, paired median differences in preconception risk and obstetric was studied a priori and via post-hoc analysis. Baseline preconception and obstetric risk indices were higher among children of multiple births versus singleton birth types. Results showed the maternal preconception risk and obstetric risk median difference was significant among singleton births, males, and cases. The rank median difference differed significantly for male births and cases, but not female births or controls. This may suggest infant gender may mediate the relationship of preconception maternal health status and obstetric risk. A second possible interpretation may be that preconception health and obstetrics may independently relate

to ASD. Suggestive evidence for the latter interpretation included the finding that birth specific nuchal cord issues at delivery were more prevalent among ASD cases.

Nuchal cord issues (a condition in which the umbilical cord is fully wrapped, 360 degrees, around the neck of the fetus at time of delivery) parental self-reported responses to questions or comments about preterm labor, pitocin use, and gestational age of specific births were analyzed posthoc. The prevalence of nuchal cord issues differed significantly ($p = 0.05$) among cases (2.4 - 2.9%) and controls (8.6 - 9.8%) for both singleton and multiple birth records. Cesarean section delivery averaged 29%, but trended ($p = 0.09$) higher among cases (31%).

The median difference between preconception risk and obstetric risk appeared to co vary for the initial criteria of obstetric risk (preeclampsia and jaundice birth) as well as supplemental obstetric criteria (gestational diabetes, edema, preterm labor despite pitocin therapy, and nuchal cord issues at infant delivery). This result is in agreement with meta-analysis results of 77 studies analyzed by Duckitt and Harrison (2005). They reported preeclampsia was associated with preexisting diabetes, twin pregnancy, null parity, hypertension, anti-phospholipid antibodies, and higher body mass index and maternal age. In this study, paired maternal median differences among preconception and obstetric risk indices including supplemental obstetric risk criteria did not improve, but appeared to maintain the multicollinearity of preconception risk factors and obstetric risk indices. In addition, gravida also showed collinearly with obstetric risk.

Proposed confounding variables were studied using the proxy variables of infant respiratory distress at delivery, and the need for resuscitation during infant delivery.

These variables were proxy definitions for interrupted infant sleeping and breathing, hypothesized to reflect possible SIDS and hypoxia risk which had been previously associated with ASD risk (Burstyn et al., 2011). In this study, odds ratio for the risk of respiratory distress given male birth (either singleton birth or multiple birth delivery) was 1.25 regardless of case/control status.

Maternal gravida was shown to be higher among female controls as well as female case offspring suggesting the suggestive offspring gender effects of respiratory issues, asthmas, and smoke exposure, may not be solely associated with the number of previous pregnancies or maternal preconception health status. Feeding difficulties, whether during breast or bottle feeding practice, tended to be higher among cases and males but may also be affected by gravida.

The proposed direction of hypothesized relationships was as expected for maternal smoke behavior, prenatal vitamin use, preconception risk factors and maternal age with subsequent ASD risk in offspring. The hypothesized positive association of lactation duration to mitigate the risk of ASD was not evident based on dedicated lactation practice information. The AGRE database results suggested the multiple birth pregnancies and total number of maternal pregnancies (i.e., gravida) were significant factors in the relationship of main effect, hypothesized placental transfer related exposures such as prior maternal smoking, and possibly lactation, to subsequent ASD risk in offspring. However, the data also indicated infant gender appeared to have a significant and independent effect on infant respiratory distress and resuscitation required at delivery and ASD diagnosis as measured by ADIR criteria in the study.

Average maternal age was did not differ by gender for the combined sample of all birth types (singleton *and* multiple births); or singleton births. Further data analysis of maternal age variable indicated a distribution skew above mean for multiple male births among older mothers. Among singleton births, maternal age was significantly ($p = 0.03$) lower for controls versus cases (30.8 versus 31.1 years, respectively). Paternal age did not differ by ADIR status, gender or for birth type (singleton or multiple birth pregnancies). Box plot analysis indicated for all offspring birth types, the distribution was significantly skewed to higher maternal age for female offspring. Previous researchers found a moderate ($OR = 1.1 - 1.6$) and independent effect of parental age on the association to ASD (Gregory et al., 2013; Grether et al., 2009). Lauritsen et al. (2005) reported parental age had a reduced effect ($OR = 1.1 - 1.7$) after adjustment for zygosity and socioeconomic status. AGRE prevalence rates for preconception risk factors of maternal diabetes, hypertension, and triple screen test positive results appear similar to that reported in other U.S. autism cohorts (Gardener & Lyall, 2014; Gregory et al., 2013; Krakowiak et al., 2012; Lawrence et al., 2008). Preconception and obstetric statistics, and missing value rate in the AGRE cohort corroborated the results of Wallace et al. (2008) who used similar AGRE data sets to study ADOS case criteria. In the AGRE cohort, pre-existing, diagnosed maternal diabetes was reported for only 13 of 902 records (i.e. 1.3%) but prevalence was skewed to cases and males. Hypertension prevalence was estimated to be 8.9% in the AGRE sample with slightly higher levels in mothers of cases and male offspring. Krakowiak et al. (2012) reported preconception hypertension rates of 3.6% in a

California cohort, whereas Gregory et al. (2013) reported 5 to 7% hypertension prevalence in a North Carolina cohort.

Covariation. In this study, mothers of singleton births had lower median preconception risk factor indices, lower average maternal age, parity, gravida, and lower obstetric risk factors than children of multiple birth delivery events. But across birth type, gender, and among cases, the median difference between preconception and obstetric risk factors was stable and significant ($p = 0.05$). This result suggested preconception and obstetric risk (as originally defined and proposed to reflect preeclampsia and jaundice birth delivery) may have covaried positively with each other, consistent with prior literature (Duckitt & Harrington, 2005). Paired median maternal differences in initially defined preconception and obstetric risk indices differed significantly for male births and cases, but not female births or controls in this study. This may be an artifact of sample size effect, or may suggest infant gender and/or gravida may mediate the relationship of preconception maternal health status and obstetric risk.

Alternatively, preconception risk and obstetric risk may be independent factors which are mediated by fetal gender in the relationship to ASD risk. This study concluded obstetric risk was not a covariate in the relationship of pregnancy factors and ASD risk. However, obstetric risks (preeclampsia and/or jaundice delivery) were associated with respiratory distress at delivery. Posthoc AGRE evidence identified a broad dimensional risk effect for preconception and obstetric risk for parents who may have used fertility treatment. Birth specific obstetric risks, such as the prevalence of nuchal cord issues differed significantly ($p = 0.05$) among cases (2.4-2.9%) and controls (8.6-9.8%) for both

singleton and multiple birth records. Cohort cesarean section delivery averaged 29%, but trended ($p = 0.09$) higher among cases (31%).

Infant breathing and sleeping patterns were presumed to be study confounders, which may represent underlying congenital heart defects, asthma, allergies, symptoms of psychotherapy medication, comorbid mental health conditions, or ear infections (Hartshorne et al., 2009). Methodology and survey instruments for infant sleeping and sleep apnea traits are not well standardized (Mahoney & Caterino, 2011; Young et al., 2009). In this study, sleep disorder onset and sleep traits (unadjusted for asthma, allergies, ear infections or psychotherapy medications) did not differ by case status. Froehlich-Santino et al. (2014) reported respiratory distress, $OR = 2.29$, 95% CI [1.12-4.67] and other markers of hypoxia, $OR = 1.99$, 95% CI [1.04-3.80] were associated with increased ASD risk in males in a California cohort of 194 twin pairs.

Fetal respiratory distress, and the need for resuscitation were studied as possible proxy variables for hypoxia related risk factors associated with ASD (Burstyn et al., 2011). The AGRE data suggested odds ratio for these traits were higher among male children ($OR = 1.25$), regardless of case status. Respiratory distress was shown to be collinear with gravida, maternal age, multiple birth pregnancies, and obstetric risks. Factor analysis showed infant respiratory distress to be a critical covariate in the relationship of maternal smoking, lactation, gender to offspring ASD. However the causality and primal determinant for the covariate effect is unclear.

Serotonin and dopamine dysregulation may be associated with hypoxia; a SIDS and ASD risk factor (Previc, 2007). Atypical bronchial airway development may be

another factor in the association of hypoxia, respiratory distress, and ASD risk (Stewart & Amar, 2014). Other California cohorts have conducted respiratory distress and hypoxia were significantly associated with ASD risk (Froehlich-Santino et al., 2014). In this study there was significant correlation between antecedent infant respiratory distress and resuscitation ($p < 0.01$) for the entire sample and by ASD status, which is logical. In the AGRE cohort, male infant gender was associated with higher respiratory issues at birth, and in childhood years. Therefore the data may suggest a conceptual alignment with hypoxia related factors and SIDS as was reported by Burstyn et al. (2011) and Froehlich-Santino et al. (2014).

Plausible mechanisms. The constructs of biological susceptibility, multi-factor liability threshold, and fetal programming were used to inform the theoretical study framework shown in Figure 2. While Tsia et al. (1981) described the theorized mechanism as more complex than the classic environmental exposure-dose-disease model, the concept did not address genetic variability or temporal factors of exposures or childhood developmental risk profiles that may be associated with autism. Glasson et al. (2004) discussed genetic susceptibility as the primary etiological role of subsequent ASD in offspring. Cohen et al. (2005) argued that epigenetic reactions between exposures and gene alleles and expression mediate potential genetic susceptibilities which may be associated with ASD.

There has been no consensus on the definition of “genetic susceptibility” to ASD, and genomic studies have been inconclusive (Tjordman et al., 2014). Dodds et al. (2011) used the term to represent an ASD case subject having an affected sibling, or mother with

a history of psychiatric or neurologic condition. The use of the AGRE familial registry which contained sibling controls, and nonfamilial controls was expected to control for potential "genetic susceptibility". However, there was inadequate statistical power to analyze only family-wise associations as was done previously using the AGRE cohort (Anello et al., 2009).

The findings of this research suggest the hypothesized main effect variables, which represent periconception period (prior maternal smoking), prenatal exposures (maternal vitamin use), and early neonatal exchange (lactation) were main predictors in the relationship to offspring ASD. Temporality of exposure were presumed by variable definitions; maternal smoking prior to pregnancy, intrapregnancy maternal multivitamin use and fish intake, and lactation duration. But the causative and primal mechanisms were not more readily discerned due to lack of specificity on the initial timing of maternal vitamin use, and lack of maternal fish intake data before and during pregnancy. Further, this study did not associate genetic allele forms to maternal or infant health status or address multiple birth pregnancies, or lactation capacity as a priori variables. Infant respiratory distress at birth was presumed to be a confounding variable, but the analysis showed it was a key covariate in the predictive final model. In addition, no direct biomarker data was available to address plausibility of placental transfer mechanisms as the primary etiologic pathway associated with subsequent ASD risk in offspring.

The concept of fetal programming has been expanded to consider a dynamic interaction between fetal development and growth, and unique and fluctuating maternal health status (Finney-Brown, 2011; Lillycrop 2011; Pedersen et al., 2013). Recent

research in placental transport and physiology has provided evidence that fetal programming may provide an explanatory mechanistic framework for the trigger events and consequences which affect fetal development and ASD risk (Neggers, 2014; Pedersen et al., 2013; Salafia, 2011; Sibley, 2009).

Within the AGRE cohort, several birth-specific obstetric risks were reported which may have contributed to the statistical significance of infant respiratory distress at birth in the finalized, predictive regression equation (see Table 16). In this study infant respiratory distress was collinear with resuscitation at delivery (as expected), child gender, maternal age, multiple birth pregnancies, and obstetric risks. However, the study design could not discern whether infant respiratory distress was primarily independently associated with genetic susceptibility, periconception health, or child gender. Recent studies also report respiratory distress, hypoxia and bronchial anatomy are independent predictors of ASD risk (Froehlich-Santino et al., 2014; Stewart & Klar, 2013). However, neonatal physiology may reflect periconception health (Habek & Kovacevic, 2011). The design of this dissertation helped to prioritize temporality of fetal and neonatal exposures which may be associated with subsequent ASD risk, but the study design could not inform ASD etiology mechanisms.

It was of interest to characterize the impact of critical windows of environmental exposure affecting ASD risk in a well-characterized genome to determine baseline information as to whether these factors occur at random, contribute to some threshold level of ASD risk, or are attributed to placental transfer mechanisms. Prior maternal smoking was shown to be antecedent, and associated with higher odds ratio than lactation

practice in this study. Infant respiratory distress at delivery is likely an antecedent factor to lactation practice. Infant respiratory distress may reflect preconception, pregnancy risks, or birth specific obstetric risk. Increased understanding of preconception, prenatal, and neonatal health factors within a genetic cohort may inform ASD etiology. The investigation of relationships of these factors to the gender-risk differential in ASD remains unclear since prevailing theories of associated biological pathways dysfunctions associated with autism do not account for gender-related risk factors. The interrelationships and hierarchy of shared and unique factors that influence the gender-differential of ASD deserve further study.

Limitations of the Study

The goal of the proposed retrospective, case-control design was to articulate an exposure-timing relationship for ASD, with minimal uncertainty bias, measurement, and recall bias with regard to health behaviors such as dietary intake, maternal household smoke exposure and health status; and adjustment for unique obstetric risks which may confound the relationship of dominant risk factors of ASD, and the ASD gender differential. Archival behavioral datasets were obtained in order to ensure adequate study sample size to study ASD gender differential. Due to the nature of the sampling frame, a genetics family-based registry for children diagnosed with autism spectrum disorders, the availability of matched controls was somewhat limited and may have affected findings. Increased preconception maternal health risk factors, as well as increasing maternal age and family size (gravida) were also directionally associated with higher ASD risk. Several distal covariates were identified a priori, but the AGRE datasets included

additional factors which were shown to be covariates, and exhibited multicollinearity with proposed preconception risk factors, maternal age, and obstetric risks. Other limitations of the AGRE study and research design are described in this section.

ASD Case Definition

The original proposal intended to define positive ASD case diagnosis as ADOS score above a standard cutoff value and ADIR cutoff value (Norris, Lecavalier, & Edwards, 2012; Zerbo et al. 2013). Norris et al. (2012) compared ADOS Modules 1 and 3 among 1,409 AGRE subjects aged 3-18 years and found predictive modeling against DSM-IV criteria and the anticipated DSM-V model were affected by child age and functioning. ADOS Module 3 (fluent speech for children older than 12 years age) showed lower indices of fit and lower inter-factor correlations than Model 1 (lack of phrase speech, three word and spontaneous meaningful phrases) for an AGRE sample population of 80% male children and 20% Hispanic/Latino ethnicity. Less than 3% of AGRE recruited children represent African-American ethnicity (Hilton, et al., 2010).

Since the average age of the enrolled children (enrolled from 2000 to 2004) ranged from 9 to 10.5 years old, ADOS-G, Module 2 which measured Phrase Speech competence for children younger than 12 years of age was initially proposed to be used as the case definition criteria. While AGRE catalogued data for ADOS- Modules 3 (fluent speech for children older than 12 years of age, *n=1161 records*) Module 3 did not appear appropriate for the Affected child cohort demographics because the median age of the sample cohort in this study was 9.5 years. Norris et al. 2012 showed ADOS-Module 3 had lower correlation to DSM-IV and anticipated DSM-V criteria for the AGRE

population. Further, ADIR is considered a more strict ASD criteria (Martin & Horriat 2012). In addition, factor analysis for predictive ASD is most commonly done using the formalized interview format used to construct ADIR scores (Norris et al., 2012) because ADIR is considered a more strict ASD criteria (Martin & Horriat). Therefore it is assumed ADIR was an adequate case criteria for the dependent variable. The use of a strict definition of ASD (ADOS and ADIR scores) to assess risk metrics may help to better understand contributions of temporal preconception, pregnancy, and neonatal traits.

Smoking Variables

Direct and indirect smoke exposure was defined to minimize type I error. But the placental transport mechanism theory may also be valid for indirect (second hand) smoke exposure as well as direct maternal preconception smoking risk. The temporality and onset of smoking was vaguely defined as "prior" to AGRE enrollment versus "current". Figure 5 shows internal validity of prior smoke exposure may be suspect; conversion of data to ranked categorical results was conducted to reduce effects of misclassification bias for smoke frequency. The validity of results of this study would be enhanced by the analysis of indirect maternal smoke exposure, and/or "ever" smoked exposure, should that data also show a significant positive association with subsequent ASD among offspring in the AGRE cohort. In addition, the conversion of the survey instrument response scale from an open-text field to a continuous or ordinal scale may improve data quality and minimize potential coding and misclassification bias.

Maternal Diet Variables

The initial proposal intended to obtain information about preconception maternal diet adequacy through proxy variables for intrapregnancy multivitamin use, and the discernment of cold water and fresh fish intake on a weekly basis through dietary recall method. AGRE prevalence rate of maternal vitamin use (30%) was similar to other U.S. pregnant women cohort data; 35% (D'Angello et al., 2007) or mothers of a younger birth cohort; 23-45% (Sullentrop et al., 2006). However, the data for vitamin use was initially missing in 45% of records, and the data available on cold or fresh water fish intake (which has higher comparative levels of DHA and EPA oils versus other protein sources) was not available. Multivitamin use behavior was assumed to be similar for multiple birth pregnancies and subsequent offspring in data recoding protocol for this study; which may have introduced misclassification bias of binary responses.

The data availability on fresh fish intake was also severely limited, and the Mothers and Metals survey instrument was administered nearly a decade after families enrolled in AGRE and completed other survey instruments and data records used in this study. Therefore, there was insufficient evidence to address the proposed relationship of maternal diet adequacy with regard to lipid, protein and calcium status. Additional maternal nutritional health information including a dietary recall of dairy products, Vitamin D, and fish intake may have helped to inform the unexpected result that dedicated lactation practice at durations longer than six months was associated with increased child ASD risk in the AGRE study.

Lactation Practice

Internal validity of lactation practice and duration was complicated since the results required separate analysis of the degree of dedication to lactation, as well as lactation duration. Lactation practice and duration included responses from mothers who may have casually attempted breastfeeding, who used both breast and bottle feeding, and others who reported only dedicated lactation. However, internal validity of lactation practice by dedicated mothers showed good internal agreement for any duration and dedicated lactation duration values.

Multiple birth pregnancy events and gravida was shown to be positively associated with increased dedicated lactation practice. But the AGRE survey instruments and data did not discern the frequency or proportional use of lactation relative to bottle feeding, or ask about the age of transition from breastmilk or formula to solids foods. In addition, in this study, there was no significant association between dedicated lactation practice and intrapregnancy maternal multivitamin use. Lactation efficacy, competence, and infant nutritional status was not analyzed.

Feeding difficulty responses (binary, intervention type, and comment questions) were available for Affected Child and Unaffected Sibs with 86 or 9.5% missing values for all records within the AGRE cohort. Feeding difficulty data included only parental self-reported responses. Feeding difficulty comments were defined and coded as “gavage/tube feeding, special nipples, thick formula, special formula or multiple interventions”. Therefore, most feeding difficulty comments appeared to reflect bottle or infant formula feeding practice. However, other comments described comments that may

be related to infant physiology rather than feeding practice. Open-text comments for feeding difficulty included “tube feeding, special nipples, or special formula” ($n = 6$ counts each), “soy formula” ($n = 4$ counts), “colic .. GERD... [or] ... reflux” ($n = 7$ counts). “Colic, GERD [and] reflux” comments were mentioned in 7 records of mothers who reportedly breastfed infants. Lactation durations were not adjusted for feeding difficulty responses, since the original study design did not further qualify lactation efficacy. AGRE survey instruments and available retrospective data files did not address questions related to lactation efficacy or perception of competence by lactating mothers. That result is not unexpected since the focus of the AGRE data repositories have historically been genetics research and biospecimen markers of autism spectrum diagnosis. Therefore, while there may be risk of recall or misclassification bias, and lack of information on lactation capacity, frequency or age of transition to infant formula, other beverages, or solid foods, the results suggested lactation practice was associated with multiple birth pregnancies, gravida, respiratory distress, and ASD.

Unaffected Sibling Controls

No prior published AGRE phenotypic data has been identified with regard to studies of lactation practice and duration, parental or maternal smoke exposure or maternal diet factors associated with ASD outcome. That finding was not surprising since the focus of AGRE research has been genetic biomarkers of ASD with biospecimen samples as primary data sources. The focus on genetic research has also constrained the opportunity for AGRE case-control studies based on phenotypic database sets. Therefore unaffected siblings were often used as 'controls' within the AGRE research community.

Unfortunately the archived data did not provide more than one control record per case record. This study did not attempt to associated particular genetic alleles associated with smoking behavior as published in the literature (Grazuleiciene et al., 2009; Nijmeijer et al., 2010; Schmidt et al., 2011; Wilhelm-Benartzi et al., 2012)

In this study, despite a lower than ideal ratio of the overall number of cases to control records, analysis of nonfamilial controls and unaffected siblings which represented the control group illustrated adequate diversity and minimal selection bias. It was of interest to explore in this study, the impact of shared environmental exposures (familial members) on ASD case status. However, the survey instrument and records for unaffected siblings were not as complete and the survey instrument used for parental self-reports in the Unaffected Sibs database was not the same survey used for Affected Child questionnaire responses. And, because of a lack of multiple non-familial controls per case, the study of covariation and tests of collinearity was complicated. Future research which allows for a higher case:control matching ratio, additional recruitment of non-familial control participants, and standardized, systematic administration of the Affected Child survey instrument to all participants may improve AGRE database robustness.

Gravida

Among AGRE singleton births, gravida, or number of total pregnancies, including all non/spontaneous abortions, varied by ADIR status for all birth types ($p = 0.01$). For singleton births, control group mothers ($n = 296$) had 2.5 previous pregnancies, 95% CI: [2.4, 2.7] and cases averaged 2.1 gravida, 95% C [1.9, 2.2]. Parity, or number of prior births who lived to at least 24 weeks, differed by gender for all birth types ($p = 0.04$).

Gravida was not an initially proposed covariate, but was indicated in this study to be positively associated with increasing prior smoke frequency and prevalence, dedicated lactation practice and duration, maternal age, female offspring, and control case status. Gravida and birth order were not identified a priori but could have been anticipated given the familial nature of the AGRE recruitment and registry. The use of assisted reproductive technology by parental age was also not controlled in this study.

Martin and Horriat (2012) studied ASD symptom severity across birth order in an AGRE cohort as an indication of shared environmental factors which may affect ASD etiology. ASD severity was measured by verbal and nonverbal cognitive and repetitive behavior tests for 346 sibling-pairs. They reported first affected children had greater severity of ASD symptoms particularly if girls were the first-born child. Declines in both verbal and nonverbal communication were demonstrated with second affected children within a given family.

Previously in the AGRE cohort, Lee et al., (2008) reported a trend toward higher ASD among singletons *and* multiple births born in April, June, and October. ASD concordant multiple births were higher in March, May, and September. The authors concluded nonheritable factors during the pre or perinatal period influenced ASD risk and gender differential ASD risk. Confounding factors (beyond preconception health and two obstetric risks) were not adjusted for maternal age.

AGRE parental age cohorts had been previously investigated in the relationship of offspring sex ratio and ASD (ADIR and ADOS criteria) using five-year maternal age cohort intervals (< 30, 30-34, 35-39, 40+) , and 393 affected children. Among 320 males

and 73 affected females, the affected child ratio of males to females was not associated with maternal or paternal age despite similar exclusion criteria used in this study. While, the researchers may not have adjusted for ADOS module, gravida (prior pregnancies including all abortions), multiple births, or parity (number of prior births surviving to at least 24 weeks age) in their study, the overall lack of evidence of a relationship of parental age and ASD mirrors the results of this study. Anello et al. (2009) investigated the relationship of offspring sex ratio and ASD using a generalized linear mixed model to control for sibship membership by treating all affected children from one family as a cluster and designating a random, family-wise coefficient (Anello et al.). Increasing paternal age was shown to reduce the AGRE cohort male:female ratio of offspring ASD diagnosis. The authors did not disclose whether the family-wise coefficient was adjusted for parity, gravida, or birth order.

Cantor et al. (2007) earlier studied the relationship of AGRE paternal age in first-born ASD cases as defined by ADIR scores for nonHispanic families for mothers less than 36 years old. Among the 312 families and 137 children, none of the fathers were older than 50 years but the data suggested a trend toward higher risk of ASD with increasing paternal age. In this study, the AGRE sample of Affected Child and Unaffected Sib records contained 20 records of extreme paternal age and 5 records of extreme maternal age. These extreme records (Quartile 3+ 1.5 times interquartile range) were all associated with cases (children with ADIR scores of one). In this current study, extremely low maternal ages ($n = 2$) and paternal ages ($n = 3$) were also identified and associated with case records. However, the mean and median parental ages in this study's

AGRE sample matched that reported in Cantor, et al., (2007). In this study parental age covariate was not adjusted for gravida, parity, or assisted reproductive technology use.

Recommendations

The conceptual framework adapted for this study, shown in Figure 2 hypothesized the plausible mechanisms of placental transfer and multiliability thresholds, and fetal programming may be the main effect relationship to subsequent ASD risk in offspring. The assumed framework attempted to account for temporality of risk factors, in preconception, conception, and neonatal phase. Adequate sample size in the retrospective case-control design was obtained to study the gender effect of ASD and the ASD gender differential. However, this AGRE study did not control, fully address, or adjust for potential confounding variables or use the initially proposed "strict" definition of ASD (ADOS and ADIR score criteria). Internal validation, adequate matching among cases and controls, and database robustness were other limitations identified which may provide additional insight into ASD risk profiles and autism etiology.

The key findings of this study showed prior (periconception and prenatal) maternal smoke exposure was significantly associated with high odds of subsequent offspring ASD diagnosis by ADIR score criteria. While this study used three estimates of maternal smoking, considerable variability was illustrated in daily smoke frequency responses (see Figure 5). The use of a continuous or ordinal response scale in subsequent studies may improve data quantification with regard to daily maternal smoke exposure risks. With specific relevance to the AGRE data collection, because of the significant relationships between prior maternal smoking behavior and offspring ASD, it may be of

interest to determine whether there is a relationship between particular genetic alleles previously associated with maternal smoking and offspring ASD (Grazuleiciene et al., 2009; Nijmeijer et al., 2010; Wilhelm-Benartzi et al., 2012) as shown in Table 3. Among AGRE offspring whose mothers reported smoking, the association of genes levels and expression associated with gender-differentiated xenobiotic clearance in rodent models (i.e. PON1, CYP and GST genes) may be of future research interest to further investigate the ASD gender differential (bin Ali et al. 2003; Thomas-Moya et al., 2006).

AGRE data availability and quality of maternal diet factors (fish intake, nutrient adequacy, multivitamin use, dairy, Vitamin D, lipid and protein intake) was less than ideal. Fish intake data was collected 8 to 10 years after initial enrollment, and collected for other research purposes (i.e., Metals and Mother survey instrument). Additional information regarding maternal health status before and during conception and pregnancy may improve the ability to discuss plausible mechanisms for placental transfer, multivitamin use, and fetal programming. Within the AGRE data collection, the relationship between maternal multivitamin use and offspring ASD as affected by COMT, MTHFR and CBS genetic alleles may be studied to reproduce the results of Schmidt et al. (2011) who used a different but overlapping cohort.

The results showed unexpected results for the direction and magnitude of the relationship of maternal self-reports of dedicated lactation practice and duration and ASD risk. The unexpected result may be associated with recall bias, or scaling-effects due to the use of open-text field duration questions. Conversely, the unexpected positive association of lactation to ASD risk should be re-analyzed with adjustment of metrics for

lactation competency, other infant beverage and food intake, maternal infant status, and validation of lactation practice efficacy.

The use of the AGRE genetics database archival data provided an opportunity to explore behavioral traits which may supplement genetic and epigenetic studies using this population. However the datasets were somewhat restricted in the ability to capture additional nonfamilial control sample data records. In addition survey instruments differed among case and control questionnaires, even for offspring from the same mother's history profile. This complicated data comparability among cases and controls. Therefore, while the target sample size of female case and control records were obtained, the extent of missing, incomplete and blank values for key variables, such as maternal health status and nutritional status factors, were widely unavailable.

Infant gender-stratified results showed a statistical trend ($p = 0.10$) for prior maternal smoking behavior and ASD for boy and girls when analyzed separately or in combination. However, due to the small number of female children ($n = 177$) in the AGRE sample, the ability to identify statistical significance for gender stratified analysis was limited (see Table 13). However, infant respiratory distress was found to be a key risk factor and covariate among male children, regardless of case/control status. Additional research with larger sample sizes is needed to better inform the ASD gender differential to provide insight into primary prevention strategies.

Respiratory distress at birth was a covariate, rather than a confounder in the relationship of pregnancy factors to ASD risk. Multiple birth deliveries, gravida, maternal age, and obstetric risk criteria illustrated colinearity with respiratory distress at birth.

These covariates also were associated with maternal smoking and dedicated lactation practice. Inclusion of respiratory distress improved predictive relationships of pregnancy factors to ASD.

There was insufficient AGRE evidence to determine whether infant respiratory distress was an antecedent proxy variable for ASD risk as previously proposed by Burstyn et al., (2011). There was also insufficient evidence to determine whether infant respiratory distress was a precedent outcome of maternal smoking during pregnancy. There was significant evidence ($p = 0.03$) respiratory distress was associated with birth specific obstetric complications of preeclampsia and jaundice. Future AGRE database studies should consider a priori sample size adjustment of proposed ASD risk factors for multiple birth deliveries, gravida, and parity.

Preconception health risk factors were defined in this study to include pre-existing diabetes, hypertension, anemia, low vitamin B, folate/neural tube risk, albuminuria. Data for preconception and health risk factors among records for affected children were fairly thorough. With regard to preconception health and reproductive risk factors, the maternal self-reported data indicated fewer than one-third of abnormal triple screen result were followed up with amniocentesis testing during pregnancies. This finding, if substantiated by physician report, may suggest more intensive follow-up may improve pregnancy outcomes or risk diagnosis for obstetric complications. Posthoc analysis indicated 10 broadly defined obstetric and preconception risk factors tended to be associated with fertility treatments for both cases and controls. Thus, adjustment of preconception factors

for assisted reproductive technology in future studies may improve predictive relationships of these and other factors to ASD risk.

Temporality of exposures associated with ASD showed maternal prior smoking was antecedent to infant respiratory distress at delivery. Respiratory distress at delivery was identified and defined as antecedent to dedicated lactation practice. These exposure relationships to ASD may be linked to in utero fetal hypoxia and respiratory distress at birth, and/or multiple birth deliveries, gravida, and/or perhaps prenatal maternal nutritional status.

Plausible relationships between maternal smoke exposure and offspring ASD, the potential association of infant respiratory distress and maternal smoke exposure, and the unexpected association between dedicated lactation and ASD risk suggests additional primary and secondary reproductive health interventions focused on optimal reproductive maternal health status before, during and after pregnancies would be helpful. Access to reproductive health education, smoking cessation, family planning, preconception health and maternal and child nutritional education programs as well as lactation education, awareness and competency building programs are additional public health implications associated with results of this study.

Social Change Implications

The concepts of placental transfer and multiliability thresholds as plausible mechanisms which may explain the risk of ASD in offspring (Neggers, 2014; Pedersen et al., 2013; Salafia, 2011; Sibley, 2009) have several implications for family planning, reproductive health education, medical and obstetric care and breastfeeding promotion. In

this AGRE study using phenotypic data for a family-based genetics registry, family size, multiple birth events, and male fetus appear to be related to high risk factors for infant respiratory distress and subsequent ASD diagnosis. Maternal smoking behavior prior to the pregnancy or fetal delivery, and dedicated lactation practice was also positively associated with ASD risk in the AGRE sample. These factors are controllable decisions related to behavior and education, rather than genetic risks. The temporality of these factors suggest primary reproductive health programs may influence the subsequent diagnosis of offspring ASD as primary, rather than tertiary ASD mediation programs. Investment in maternal and child health may also have broader benefits beyond ASD risk and diagnostic ASD outcomes.

In 2002, between 23 to 34% of SIDS deaths among U.S. infants was attributed to prenatal smoking (CDC, 2013). Unfortunately, smoking prevalence before, during, and after pregnancy in the U.S. has not changed or been reduced over the period 2000 to 2010 (CDC, 2013). Thus, reproductive health education programs must focus on smoking cessation. Prior periconception maternal smoking may affect fetal hypoxia risk and respiratory distress (Froehlich-Santino et al. 2014; Habek & Kovacevic, 2011). Maternal nutritional and placental health may also likely be adversely affected by preconception maternal smoke exposure; via direct or indirect maternal smoke exposure.

Placental transfer and placental dysfunction, maternal diet, fatty acid, and fat soluble vitamin status (i.e. vitamin D) research are active research areas associated with “fetal programming” hypotheses (Baker et al., 2010; Novak & Innis, 2012; Robinson et al., 2010; Saugstad, 2011). Low maternal vitamin D levels in the third trimester of

pregnancy have also been associated with risk of subsequent autism (Dietert et al., 2011). Therefore, maternal nutritional adequacy likely affects birth outcomes, fetal health, and subsequent risk of ASD diagnosis in offspring.

Optimized primary reproductive health may benefit from greater focus on family planning, birth spacing, risk-benefit analysis of assisted reproductive technologies, and preconception health status. In this study, abnormal triple screen follow-up consultation was reportedly less frequent for affected male fetuses (52.5%) than affected female fetuses (58.5%), suggesting disciplined prenatal monitoring may be critical. In this study, infant males, regardless of case/control status appeared to be at risk for respiratory distress and resuscitation at delivery.

Data for maternal triple screen test, which tested for alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG), and estriol (uE3) were available in the Affected Child database. This triple marker tests is a prenatal screen, typically done at 15 to 20 weeks gestation, to test for birth defects, including Down's syndrome, spina bifida, and anencephaly (American Pregnancy Association, 2006). Positive maternal triple screen test may indicate Down's syndrome, risk of spina bifida or low vitamin B/folate, or abnormal kidney or albuminuria diagnosis. Preconception health, increased maternal age, preeclampsia and jaundice birth were suggestively associated with higher ASD risk, as were birth-specific obstetric issues such as nuchal cord issues which may induce hypoxia or fetal respiratory distress. Therefore, comprehensive reproductive health, including fetal monitoring, and optimized delivery procedures, may mediate gender-associated risks associated with subsequent ASD risk.

Maternal prior smoking behavior, prenatal dietary status, and gravida may affect placental transfer of nutrients in utero, as well as postdelivery lactation efficacy.

Lactation efficacy may also be associated with ASD risk based on the AGRE responses to "feeding difficulties", particularly among male infants. Birth facilities that provide recommended nutritional and medical care and postdelivery instruction for lactating mothers, and worksite lactation support programs are also strongly encouraged by Healthy People 2020 goals (DHSS, 2014).

Conclusions

In this study, using retrospective, archived phenotypic datasets for a family-based genetics data registry, several behavioral related risk factors were associated with ASD risk in a case-control study design. These behavioral risk factors are currently and readily addressed in reproductive and maternal health education guidelines focused on primary prevention education. Additional behavioral risks identified in this study are addressed in pregnancy monitoring and medical follow-up procedures for obstetric and gynecologic health before and during pregnancy. Nutritional education, and healthy balanced dietary guidelines also reinforce behavioral risk factors identified in this study. It is anticipated that a refocus on these existing, well-established and current public policy practices may have additional advantages in enhancing fetal health.

Prior maternal smoke exposure and infant respiratory distress at birth, and lactation was associated with subsequent risk of ASD diagnosis in offspring. Maternal and household smoke exposure is a controllable, health behavior which affects infant SIDS risk as well as ASD risk for children. More than one-fourth of infant deaths are

SIDS related, but the prevalence of smoking before, during and after pregnancy has not declined over the period of 2000 to 2010 (CDC, 2013).

Multiliability threshold and/or placental transfer construct theories may explain plausible mechanisms associated with increased ASD risk in offspring for variables in this study. Temporality of mechanisms may affect ASD risk development. It was shown prior maternal smoking was antecedent to infant respiratory distress. Respiratory distress at birth was identified as an antecedent to dedicated lactation which significantly related to subsequent ASD risk.

A better understanding of the factors that affect ASD and the ASD gender differential may inform reproductive risk profiles and public health policy efforts aimed at women of childbearing age. In this study, the exposure-timing risk relationships were suggestively related in meaningful, plausible, temporal ways. It is my hope study outcomes may reinforce the need for primary reproductive health care, and challenge the immoderate focus on tertiary care, mediation, and management of ASD symptomology toward primary preventive reproductive and prenatal health care.

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Appendix A. Diagnostic Criteria for Clinical Autism Spectrum Disorder

Autistic Disorder— DSM-IV Diagnostic Criteria (diagnostic code 299.00)

A total of six (or more) items from A, B, and C, with at least two from A, and one each from B and C:

A. Qualitative impairment in social interaction, as manifested by at least two of the following:

B. Qualitative impairments in communication as manifested by at least one of the following:

1. Marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction
2. Failure to develop peer relationships appropriate to developmental level **
3. A lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)
4. Lack of social or emotional reciprocity
5. Delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative communication modes such as gesture or mime)
6. In individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
7. Stereotyped and repetitive use of language or idiosyncratic language
8. Lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level
9. Encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

- 10. Apparently inflexible adherence to specific, nonfunctional routines or rituals
- 11. Stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting or complex whole-body movements)
- 12. Persistent preoccupation with parts of objects

C. Restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:

Delays or abnormal functioning in at least one of the following areas, with onset prior to age three years: 1) social interaction, 2) language as used in social communication, or 3) symbolic or imaginative play

Modified Checklist for Autism in Toddlers (M-CHAT) can be used by parental self-report as an initial screener, but is typically validated by formal ADI-R and ADOS clinical diagnosis meeting ICD-9 criteria.

** It is expected that the *DSM-V*, planned to be finalized in 2013 will further modify the criteria and broaden the scope and definition compared to *DSM-IV* (American Psychiatric Association, 2012).

The revised *DSM-V* criteria are intended to reflect patient history rather than depending on behaviors observed during clinical assessment.

Social behavioral domain criteria will be altered from "...failure to develop peer relationships and abnormal social play.." to include "... difficulties adjusting behavior to suit different social contexts..." (American Psychiatric Association, 2012).

Appendix B: AGRE Lifestyle Protocol Survey

(selected abstracts of questions referenced in Table 4)

AGRE Lifestyle: Online System for Clinical Research (OSCR)

Respondent Instructions:

We are going to ask you some questions about your lifestyle.

Section A: Tobacco

1. Have you ever smoked cigarettes?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "YES" to question 1, go to question 2, otherwise, skip to question 10.

2. Have you ever smoked regularly? By regularly, I mean one or more cigarettes per day on most days

for at least six months.

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "YES" to question 2, go to question 3, otherwise, skip to question 4.

3. When did you first smoke regularly?

_____ yrs. _____ mos.

- Don't Know**

4. During the three months before your pregnancy with temp test until now, did you smoke cigarettes?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "YES" to question 4, go to question 5, otherwise, skip to question 7.

5. During which months, starting with three months before pregnancy and extending through birth (and breastfeeding, if applicable), did you smoke?

- 3 months before pregnancy
- 2 months before pregnancy

- 1 month before pregnancy
- 1 month pregnant
- 2 months pregnant
- 3 months pregnant
- 4 months pregnant
- 5 months pregnant
- 6 months pregnant
- 7 months pregnant
- 8 months pregnant
- 9 months pregnant
- Breastfeeding
- Don't Know**

6. During which years of the child's life did you smoke?

- Less than 1 year old
- 1 year old
- 2 years old
- 3 years old
- 4 years old
- 5 years old or later
- DID NOT SMOKE AFTER CHILD'S BIRTH

7. During the time in which you smoked, about how many cigarettes did you smoke a day?

_____ # cigarettes per day

- Don't Know**

10. Have you ever at any time used other tobacco products such as a pipe, snuff, cigar, or have you ever used a nicotine patch?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "YES" to question 10, go to question 11, otherwise, skip to question 15.

11. What did you use? _____

- Don't Know**

12. During the three months before your pregnancy with temp test and extending through birth (and breastfeeding, if applicable), did you use this product(s)?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "YES" to question 12, go to question 13, otherwise, skip to question 15.

13. During which months did you use the product

- 3 months before pregnancy
- 2 months before pregnancy
- 1 month before pregnancy
- 1 month pregnant
- 2 months pregnant
- 3 months pregnant
- 4 months pregnant
- 5 months pregnant
- 6 months pregnant
- 7 months pregnant
- 8 months pregnant
- 9 months pregnant
- Breastfeeding
- Don't Know**

14. During the months in which you used the product, about how many times a day did you use it? _____ # times a day

15. During the index time until now, did you live with anyone who smoked cigarettes?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "YES" to question 15, go to question 16, otherwise, skip to question

18.

16. How many people living in your home smoked inside your home?

_____ # people

- Don't Know**

17. During which months, starting with three months before pregnancy until now, did you live with that person (or those people - if more than one)? (Same categories as used in question 13)

Appendix C. AGRE Medical History- Child Survey Section A, B, and C

(selected abstracts of questions referenced in Table 4)

AGRE Medical History –CHILD: OSCR

Respondent Instructions:

We are going to be asking you about your child's entire medical history, starting with his/her specific pregnancy.

Please consult your medical records to help ensure accuracy. You might find it helpful to have these records in front of you while completing the survey.

Section A: Medical History

Prenatal History Section:

23. Did the child's mother have a blood test to check for neural tube defects (spinal bifida) or Down Syndrome (a.k.a. Alpha-fetoprotein or triple screen test)? *(THIS IS A STANDARD SCREEN)*

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "YES" to question 23, go to question 24, otherwise, skip to question 25.

24. Were the results abnormal?

- No
- Yes (Please describe: _____)
- Don't Know**

At ANY time in this pregnancy, did the birth mother have any of the following health problems:

48. Anemia (low iron in the blood)

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 48, go to question 49, otherwise, skip to question 50.

49. In what trimester did this occur? (CHECK ALL THAT APPLY)

- 1st
- 2nd
- 3rd
- Don't Know**

58. Albuminuria

- No
- Yes
- DK**

INSTRUCTIONS: If you answered "Yes" to question 58, go to question 59, otherwise, skip to question 60.

59. In what trimester did this occur? (CHECK ALL THAT APPLY)

- 1st
- 2nd
- 3rd
- Don't Know**

90. Preeclampsia/eclampsia/toxemia

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 90, go to question 91, otherwise, skip to question 93.

91. In what trimester did this occur? (CHECK ALL THAT APPLY)

- 1st
- 2nd
- 3rd
- Don't Know**

92. Were any medications taken?

- No
- Yes
- Don't Know**

Medications/Supplements during THIS pregnancy

121. During THIS pregnancy did the birth mother take prenatal vitamins?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 121, go to question 122, otherwise, skip to question 124.

122. Did she take them continuously throughout the pregnancy?

- No
- Yes
- Don't Know**

123. In what trimester did she take them?

- 1st
- 2nd
- 3rd
- Don't Know**

124. During THIS pregnancy did the birth mother take any other nutritional supplements continuously?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "No" to question 124, go to question 125, otherwise, skip to question 126.

125. Supplement

In which trimester(s) did this occur?

(CHECK ALL THAT APPLY) _____

- 1st
- 2nd
- 3rd
- Don't Know** _____

- 1st
- 2nd
- 3rd

Don't Know

- 1st
- 2nd
- 3rd

Don't Know

_____ (Same categories as above)

Now we are going to ask questions regarding labor, delivery and newborn information for the child:

130. At the time that the child was born, how many weeks had the birth mother been pregnant (gestational age)? _____ weeks

Don't Know

131. How did labor start?

- Spontaneous
- Induced by physician
- Never happened (Planned C-Section preceded labor)

Don't Know

INSTRUCTIONS: If you answered "Induced by physician" to question 131, go to question 132, otherwise, skip to question 135.

132. Did the doctor put a gel type medication directly onto the cervix?

- No
- Yes

Don't Know

133. Did the doctor give her a medication through an IV (like pitocin)?

- No
- Yes

Don't Know

134. Why did the doctor need to do this? (CHECK ALL THAT APPLY)

- Water broke (membranes ruptured) prematurely (too early)
- Water broke and the contractions didn't get started for more than 24 hours
- Baby was past its due date
- Convenience
- Other _____

Don't Know

135. Did the doctor need to restart or speed up the birth mother's labor with pitocin?

- No
- Yes

Don't Know

INSTRUCTIONS: If you answered "No" to question 135, go to question 136, otherwise, skip to question 138.

136. Why did the doctor need to do this? (CHECK ALL THAT APPLY)

- Contractions stopped
- Baby didn't come down the birth canal
- Cervix didn't dilate fast enough
- Other _____

Don't Know

137. Did the doctor break the mother's water to induce or speed up the labor?

- No
- Yes
- Don't Know**

158. Did this baby stay in a neonatal intensive care unit (NICU)?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "No" to question 158, go to question 159, otherwise, skip to question 162.

159. For how long?

_____ days OR _____ hours

- Don't Know**

160. Was the baby on a respirator (ventilator)?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "No" to question 160, go to question 161, otherwise, skip to question 162.

161. For how long?

_____ hours OR _____ days

- Don't Know**

164. How many days or hours TOTAL did this baby stay in the hospital? (*after delivery up until discharge, including the neonatal ICU*)

_____ days

- Don't Know**

165. How many days did the birth mother stay in the hospital?

_____ days

- Don't Know**

166. Did the baby have any diagnosed medical problems in the newborn period? (**0-30 days of life**)

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 166, go to question 167,

otherwise, skip to Section B.

167. What type? (CHECK ALL THAT APPLY)

- Head deformities
- Body deformities
- Limb deformities
- Heart deformities
- Kidney deformities
- Stomach/intestine problems
- Other deformities _____
- Sepsis (bacterial blood infection)
- Jaundice, hyper bilirubinemia, yellow skin

INSTRUCTIONS: If "Yes":

168. What treatment was given? (CHECK ALL THAT APPLY)

- None
- Phototherapy (special lights)
- Exchange transfusion (blood transfusion)
- Don't Know**
- Anemia
- Seizures

INSTRUCTIONS: If "Yes":

169. What type?

- Grand mal/Generalized tonic-clonic
- Petit mal/absence
- Infantile spasms
- Complex partial
- Multiple types
- Other _____
- Don't Know**
- Meningitis
- High Fever (>38.5 C or 101.5 F)
- Other: _____

Respondent Instructions:

We are going to be asking you about your child's entire medical history, starting with his/her specific pregnancy.

Please consult your medical records to help ensure accuracy. You might find it helpful to have these records in front of you while completing the survey.

Section B: Early Development

Now we are going to ask some questions regarding the child's EARLY DEVELOPMENT

(For the purposes of our survey please regard "early development" as the **first 12 months of life**)

1. Did the birth mother breastfeed OR pump milk to the baby?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 1, go to question 2, otherwise, skip to question 10.

2. How long was breast milk the only source of the baby's nutrition? (*that is, without supplemental formula or solid food*)

_____ months _____ weeks

- Don't Know**

3. What was the baby's age in months at the start of breast milk feeding?

_____ months _____ weeks

- Don't Know**

4. How old was the child in months when s/he received the last/final breast milk feeding?

_____ months _____ weeks

- Don't Know**

5. Did the child have any difficulty latching onto the breast?

- No
- Yes
- Don't Know**

6. Did the birth mother ever smoke while the child was on breast milk?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 6, go to question 7, otherwise, skip to question 8.

7. How many packs were smoked per week?

_____ packs

- Don't Know**

10. Did the birth mother ever use any medication (including over the counter, prescription or vitamin supplements) while the child was on breast milk?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 10, go to question 11, otherwise, skip to question 16.

11. Vitamins - please CHECK ALL THAT APPLY and **specify how long it was taken (in months).**

Vitamin type How long (in months) it was used?

Multivitamins
_____ months

Don't Know

Vitamin A
_____ months

Don't Know

Vitamin B6
_____ months

Don't Know

Vitamin B12
_____ months

Don't Know

Folic Acid
_____ months

Don't Know

Vitamin C
_____ months

Don't Know

Vitamin D
_____ months

Don't Know

Vitamin E
_____ months

Don't Know

Iron

_____ months

Don't Know

Calcium

_____ months

Don't Know

Zinc

_____ months

Don't Know

12. Other supplements (e.g. *Slim Fast, Instant Breakfast, protein powder, brewer's yeast*)

Supplement **How long (in months) it was used?** _____

_____ months

Don't Know

16. Was the child ever fed formula?

No

Yes

Don't Know

INSTRUCTIONS: If you answered "Yes" to question 16, go to question 17, otherwise, skip to question 19.

17. How long was formula the only source of the baby's nutrition? (*that is, without solid food*)

___ weeks

Don't Know

18. What type of formula?

Soy based (*Enfamil Prosoabee LIPIL, Isomil, Isomil Advance, Isomil DF, Good Start Supreme Soy*)

Cow's milk (*Enfamil LIPIL, Enfamil Gentlease LIPIL, Enfamil w Iron, Similac Advance, Similac w Iron, Good Start Supreme, Good Start Essentials and NA*)

Elemental formula (*Nutramigen, Pregestamil, Alimentum*)

Lactose free formula (*Lactofree, Similac Lactose free*)

Formula supplemented with DHA/ARA (*Enfamil Lipil, Similac Advance, Nestle Good Start Supreme DHA ARA*)

Premie formula (*Enfacare LIPIL and Neosure Advance*)

Other (Please specify: _____)

Don't Know

19. Did you have any difficulty with feeding (breast or bottle)?

No

Yes

Don't Know

INSTRUCTIONS: If you answered "Yes" to question 19, go to question 20, otherwise, skip to question 24.

20. Did the baby have poor suck?

No

Yes

Don't Know

21. Did the baby require special feeds?

No

Yes

Don't Know

INSTRUCTIONS: If you answered "Yes" to question 21, go to question 22, otherwise, skip to question 24.

22. What interventions were used? (CHECK ALL THAT APPLY)

Thickened liquid

Special nipples

Gavage (force feeding)

Other _____

Don't Know

23. When did this happen?

From ____mos to ____mos

Don't Know

31. How was the baby's early temperament?

Normal

Difficult/Irregular

Easy/Passive

Don't Know

32. How was the baby's early sleep pattern?

Regular/Predictable

Irregular/Unpredictable

Don't Know

33. Did the child have any difficulties like colic or being difficult to sooth?

No

Yes

Don't Know

INSTRUCTIONS: If you answered "Yes" to question 33, go to question 34, otherwise, skip to question 36.

34. What age did it start? _____ months

Don't Know

35. What age did it stop? _____ months

Ongoing

Don't Know

36. Was the child exceptionally floppy as an infant?

No

Yes

Don't Know

37. Was the child exceptionally stiff as an infant?

No

Yes

Don't Know

38. Was the child exceptionally irritable/inconsolable as an infant?

No

Yes

Don't Know

39. Was the child exceptionally lethargic or overly sleepy as an infant?

No

Yes

Don't Know

Respondent Instructions:

Now we are going to ask you about the child's physical development and medical history (FROM BIRTH TO NOW). For each category, first assess if there are any concerns in that general area, if so, please check all the appropriate diagnoses and/or symptoms listed below it. FOR ISSUES THAT WERE NOT PRESENT AT BIRTH YOU WILL BE ASKED TO SPECIFY AT WHAT AGE IT FIRST STARTED.

Section C: Physical Development

30. **Nose/throat-***Is there any known issue/abnormality in this area?*

No

Yes

Don't Know

INSTRUCTIONS: If you answered "Yes" to question 30, go to question 31, otherwise, skip to question 32.

- Excessive snoring
- 0-11 months
- 1 year old
- 2 years old
- 3 years old
- 4 years old
- 5 years old
- 6 years old
- 7 years old
- 8 years old or older (Specify age: _____yrs)

Don't Know

N/A

- Tonsillectomy (Same categories as above)
- Adenoidectomy
- Edema (swelling caused by excess fluid)
- Stiffness

38. Pulmonary-Is there any known issue/abnormality in this area?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 38, go to question 39, otherwise, skip to question 40.

39. Abnormality (CHECK ALL THAT APPLY)

Age of Onset How long was this a regular issue?

- Shortness of breath 0-11 months
- 1 year old
- 2 years old
- 3 years old
- 4 years old
- 5 years old (cont.)
- 6 years old
- 7 years old

- 8 years old or older (Specify age: _____yrs)
- Asthma (Same categories as above)
- Recurrent pneumonias
- Chronic bronchitis

40. **Cardiovascular**-*Is there any known issue/abnormality in this area?*

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 40, go to question 41, otherwise, skip to question 42.

41. Abnormality (CHECK ALL THAT APPLY)

Age of Onset How long was this a regular issue?

- Congenital heart disease 0-11 months
- 1 year old
- 2 years old
- 3 years old
- 4 years old
- 5 years old
- 6 years old
- 7 years old
- 8 years old or

older (Specify age: _____yrs)

- Don't Know**
- 3 months or less
- 4 months to 1 year
- 1-2 years
- 3-4 years
- 5 years or longer
- Don't Know**
- Heart murmur (Same categories as above)

Severe sleep disturbance

IF YES

80. What type? (CHECK ALL THAT APPLY)

- Difficulty falling asleep
- Nighttime awakenings
- Short amount of sleep (less than 6 hours)
- Other _____
- 0-11 months
- 1 year old
- 2 years old
- 3 years old
- 4 years old
- 5 years old
- 6 years old
- 7 years old
- 8 years old or
older (Specify age: _____yrs)

- Don't Know**
- 3 months or less
- 4 months to 1 year
- 1-2 years
- 3-4 years
- 5 years or longer
- Don't Know**

85. **Psychiatric**-Have you ever been worried about the child experiencing symptoms in this area?

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 85, go to question 86, otherwise, skip to question 92.

86. Abnormality (CHECK ALL THAT APPLY) Age of Onset How long was this a regular issue?

- ADHD

IF YES **87. (CHECK ALL THAT APPLY)**

- Diagnosed with ADHD
- Trouble with attention/concentration
- Excessively distractable
- Hyperactive

- 0-11 months
- 1 year old
- 2 years old
- 3 years old
- 4 years old
- 5 years old
- 6 years old
- 7 years old
- 8 years old or older (Specify age: _____yrs)

92. **Genetic Syndromes**-*Is there any known issue/abnormality in this area of development?*

- No
- Yes
- Don't Know**

INSTRUCTIONS: If you answered "Yes" to question 92, go to question 93, otherwise, skip to question 94.

93. CHECK ALL THAT APPLY

- Fragile X
- Tuberous sclerosis
- Down syndrome (trisomy 21)
- Neurofibromatosis (type 1 and 2)
- Rett syndrome
- Angelman syndrome
- Prader Willi syndrome
- Phenylketnuria
- Williams syndrome
- Other chromosomal abnormality, disorder, or syndrome_____

94. Does the child have any other diagnosed medical conditions not previously asked about?

Appendix D. AGRE Metal and Mother's Diet Survey

(selected abstracts of questions referenced in Table 4)

Metals and Mother's Diet: OSCR

Respondent Instructions:

In this form, we are going to ask you about your diet, some products you may have used and dental work you may have had during the 3 months before your pregnancy with this child to the date of this child's birth. (Also we will include the period of breastfeeding.)

Section A: Diet

A.1 FISH

1. Starting with three months before the pregnancy with the child and extending through birth (and breastfeeding, if applicable) did you ever eat any fish (including a tuna fish sandwich, fish sticks, or any other kind of fish)?

- No
- Yes
- Don't know

INSTRUCTIONS: If you answered "YES" to question 1, go to question 2, otherwise, skip to question 9.

2. What kind(s) of fish has did you eat during THIS pregnancy? (CHECK ALL THAT APPLY)

2a. Type of Fish 2b. Time period in which you ate this type of fish?

2c. On average, how many servings per week did you eat? (Same categories for all items)

- Mackerel 1-3 months before pregnancy
- 1st trimester
- 2nd trimester
- 3rd trimester
- Breastfeeding
- Don't know
- Less than 1
- About 1
- More than 1
- Don't know
- Tilefish 1-3 months before pregnancy
- Swordfish 1-3 months before pregnancy
- Shark 1-3 months before pregnancy

- Red snapper 1-3 months before pregnancy
- Marlin 1-3 months before pregnancy
- Tuna (Canned) 1-3 months before pregnancy
- Tuna (Fresh) 1-3 months before pregnancy
- Bass 1-3 months before pregnancy
- Catfish 1-3 months before pregnancy
- Cod 1-3 months before pregnancy
- Crab 1-3 months before pregnancy
- Lobster 1-3 months before pregnancy
- Salmon 1-3 months before pregnancy (cont.)
- Trout 1-3 months before pregnancy

Other ocean fish (e.g.
Mahi Mahi, Pullock, etc.)

Please specify: _____

- Other fresh water fish (fish from lakes, ponds, rivers)

Please specify: _____

3. Did you ever eat any fish that came from a fish farm (farmed fish) during pregnancy with this child?

- No
- Yes
- Don't know

INSTRUCTIONS: If you answered "YES" to question 3, go to question 4, otherwise, skip to question 6.

4. During which time period did you eat this? (CHECK ALL THAT APPLY)

- 1-3 months before pregnancy
- 1st trimester
- 2nd trimester
- 3rd trimester
- Breastfeeding
- Don't know

5. On average, how many servings per week did you eat?

- Less than 1
- About 1
- More than 1
- Don't know

6. Did you ever eat fish that you caught or that someone else that you knew caught during your pregnancy with this child?

- No
- Yes
- Don't know

INSTRUCTIONS: If you answered "YES" to question 6 and 7, otherwise, skip to question 9.

7. During which time period did you eat this? (CHECK ALL THAT APPLY)

- 1-3 months before pregnancy
- 1st trimester
- 2nd trimester
- 3rd trimester
- Breastfeeding
- Don't know

8. On average, how many servings per week did you eat?

- Less than 1
- About 1
- More than 1
- Don't know

Appendix E. AGRE Mother's Medical History - Section A and B

(selected abstracts of questions referenced in Table 4)

Mother's General Medical History: OSCR

Respondent Instructions:

Now we are going to ask you about your history of medical conditions/issues.

Are there any known abnormality/issue in the following areas:

Please read through all the choices in each area as things may be grouped in a manner you are unfamiliar with. Please remember: did these issues occur AT ANY TIME in your life?

Section A: Medical History

46. Respiratory= *Is there any known issue/abnormality in this area?*

INSTRUCTIONS: If you answered "YES" to question 46 & 47, otherwise, skip to question 48.

49. Cardiovascular **What type? (CHECK ALL THAT APPLY) When did this develop? (Same categories per item)**

- High Blood Pressure (HTN) Infancy (0-12 months)
- Toddler (13-24 months)
- Early childhood (25 months to 59 months)
- Childhood 5-12 years
- Teenage (13-19 years)
- 20-29 years
- 30-39 years
- additional categories... ..
- 90-99 years
- Don't Know**
- Heart Attack (Myocardial Infarction [MI])
- Peripheral Vascular Disease (PVD) Infancy (0-12 months)
- Arrhythmias Infancy (0-12 months)
- Coronary Artery/Heart Disease Infancy (0-12 months)
- Other _____

55. Blood/Hematological - *Is there any known issue/abnormality in this area?*

- No
- Yes
- Don't Know**
- B12 deficiency (Same categories for onset for all survey questions)**
 - Infancy (0-12 months)
 - Toddler (13-24 months)

- Early childhood (25 months to 59 months)
- Childhood 5-12 years
- Teenage (13-19 years)
- 20-29 years
- 30-39 years
- additional categories... ..
- 90-99 years
- Don't Know**
- Hemalytic**
- Other _____

58. Other Medical issues we have not asked about - *Is there any known issue/abnormality in this area?*

- No
- Yes
- Don't Know**

INSTRUCTIONS: If YES list

Section B: Reproductive System and Pregnancy History

Respondent Instructions:

Now we are going to ask you some general questions regarding your reproductive system and pregnancy history

Reproductive History:

Pregnancy history:

7: How many times have you been pregnant? _____ # PREGNANCIES

- Don't Know**

INSTRUCTIONS:

Please count EVERY time you have been pregnant (include pregnancies that ended in live birth, still birth, abortion, miscarriage, tubal / ectopic, molar). This would include pregnancies from relationships other than your current one, and also if you are pregnant now

Now we are going to ask you about each of your pregnancies (i.e. what the outcome was, and when did that outcome occur). For instance, if your first pregnancy was a miscarriage you would answer Preg #1 outcome =miscarriage and it occurred at 10 weeks. If your third pregnancy was a live birth that was delivered prematurely you would choose Preg #3 outcome=live birth and that it occurred at 32 weeks

Note: If one pregnancy resulted in multiples (e.g., twins), please list them separately, by order of pregnancy outcome (e.g., list earliest first). For example, if twins, one miscarried and one live birth, the miscarried one would be listed first

List #1 (Use for question 8 - 18)

Outcomes of pregnancies.

Code 1 – Live Birth

Code 2 – Stillbirth

Code 3 – Chose to terminate pregnancy

Code 4 – Miscarriage

Code 5 – Tubal pregnancy

Code 6 – Molar pregnancy Define

Code 7 – Current pregnancy

Please note: (Answering in # of weeks is preferred, but if you are unsure please answer during which trimester)

Pregnancy 1

8. What was the outcome? _____

9. When did this outcome occur? (# weeks) _____

Unsure # weeks

If UNSURE

What trimester?

1st

2nd

3rd

Don't Know

Pregnancy 2

10. What was the outcome? _____

N/A

11. When did this outcome occur? (# weeks) _____

Unsure # weeks

If UNSURE

What trimester?

1st

2nd

3rd

Don't Know

Additional Pregnancys (Same questions and response categories as above items)

Section C: Family Medical History

Respondent Instructions:

Now we are going to ask you some questions about your extended families medical histories:

1. Did either of your biological parents have any of the following health issues?

Autism, Asperger's, Pervasive Developmental Disorder, Rett Syndrome, Fragile X, Tuberous Sclerosis, Neurofibromatosis, Prader Willi or Angelman Syndrome, Down Syndrome, Phenylketonuria (PKU), Chromosomal abnormalities (deletions, duplications) or Other genetic syndrome (e.g. Sotos syndrome, Joubert syndrome, Williams syndrome)?

- No
- Yes
- Don't Know**

2. Health Issue

(CHECK ALL THAT APPLY) **Was this person diagnosed?**

Was this person hospitalized for this issue?

- Autism Diagnosed Hospitalized
- Asperger's Diagnosed Hospitalized
- Pervasive Developmental Disorder (PDD)
- Diagnosed Hospitalized
- Rett Syndrome Diagnosed Hospitalized
- Fragile X Diagnosed Hospitalized
- Tuberous Sclerosis Diagnosed Hospitalized
- Neurofibromatosis Diagnosed Hospitalized
- Prader Willi or Angelman Syndrome Diagnosed Hospitalized
- Down Syndrome Diagnosed Hospitalized
- Phenylketonuria (PKU) Diagnosed Hospitalized
- Chromosomal abnormalities (deletions, duplications)
- Diagnosed Hospitalized
- Other genetic syndrome (e.g. Sotos syndrome, Joubert syndrome, Williams syndrome)

INSTRUCTIONS: If YES specify:

-
- Diagnosed Hospitalized.
 - Don't Know**

Curriculum Vitae

DONNA L. SULLIVAN**EDUCATION**

PhD Public Health- Epidemiology	February 2015
Walden University, Minneapolis, MN (on-line program)	
Dissertation Topic: "Temporality of Risk Factors and the Gender Differential Related to Autism Spectrum Disorder Diagnosis"	
Masters of Business Administration (Marketing, Finance)	1993
Saint Louis University, St. Louis, MO	
M.S. Food Science (Applied Organic Chemistry; Flavor and Proteins)	1990
University of Minnesota, St. Paul, MN	
B.A. Chemistry with Distinction	1982
University of Minnesota, Morris, MN	

PROFESSIONAL EXPERIENCE**Teaching Experience:**

Applied graduate studies	2008 – 2014
Assisted corporate Training and Quality staff and agent personnel	2012 – 2013
Informal mentor and peer-coach to other NORC employees	2010 – 2015
American Public Health Association- Education Committee member	2009 – 2010
Teaching Assistant in mixed classroom, full-time early childhood site	2008 – 2010
Lab Technician supervision in test analytics and statistics at Reliv	2007 – 2008
Sales force Training & Marketing Mentor	2004 – 2006
Corporate Toastmaster Education Committee Chair	2003
Workshop Tutor/Teaching Assistant, University of MN	1984 – 1985
Lab Tutor- Organic, Quant/Qual Chemistry and Biology, University MN	1981 – 1982
Residential Dormitory Advisor (co-ed by floors), University MN	1981

Research Experience:

Quality Measurement Manager	2012 - 2013
UniGroup, Inc., Fenton, MO	
<ul style="list-style-type: none"> Reviewed survey instrument and sampling methods for transition to new vendor Monitor data collection, coding, cleaning, analysis, and performance reporting 	
Field Interviewer & Survey Validation Manager	2010 - 2015
National Opinion Research Center at the University of Chicago	
<ul style="list-style-type: none"> Identified, qualified, implemented federal surveys with high fidelity and quality Recorded responses, transmitted, coded data records and met project quota 	

Director Nutritional Tech Service

2007 - 2008

Reliv International, Inc., Chesterfield, MO

- Directed R&D and developed a retail dietary supplement for clinical trial phase
- Routinized material validation, guided quality and regulatory compliance teams
- Identified and summarized scientific evidence for structure-function substantiation

Strategic Industry Analyst - Global Soybean Crop

2001 - 2004

Solae Company, St. Louis, MO

- Food industry consultant to support DuPont's global acquisition team.
- Summarized major sources & uses of edible and non-edible soy materials
- Quantified volume, profit and health impact of 2 proposed FDA health claims

Project Leader- Product Development

1986 - 1993

Ralston Purina: Protein Technologies International, St. Louis, MO

- Laboratory and production scale-up of several protein technologies

M.S. Thesis

Defended 1990

Maillard browning in stored sugar-amine model systems at ambient temperatures.

Sugar degradation, 2-fufuraldehyde and maltol formation were most evident reactions.

Junior Scientist- Environmental Chemistry

1982 - 1984

USDA, Soil Science Dept. at University of MN, St. Paul, MN

RESEARCH INTERESTS

Behavioral and Community Health Education; Maternal and Child Public Health;
Molecular, Nutritional, Environmental, Occupational, and Social Health Epidemiology

PROFESSIONAL ASSOCIATIONS

American Public Health Association
Association of Univ. Technol. Management
American Association of University Women

Institute of Food Technologists
Toastmasters International
American Marketing Association