Investigating Early Risk Factors for Autism Spectrum Disorder

by

Lisa Marie Unwin

BA (Hons)

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School of Psychology & Telethon Kids Institute, University of Western Australia
Abstract

The most frequent age for diagnosis of Autism Spectrum Disorder (ASD) is between 2 and 5 years of age, with an average diagnostic age in Australia of 4.5 years (Bent, Dissanayake, & Barbaro, 2015; Dobkins, Akshoomoff, Carver, Dorhmann, & McCleery, 2008). Diagnosis in Australia is occurring long after the early signs of ASD first emerge at around 6-12 months of age. Consequently, there are missed opportunities for therapies to commence when neuroplasticity is greatest. The overarching objective of this thesis was to investigate potential pre- and postnatal indicators of ASD risk in a prospective cohort of siblings of children with ASD (‘high-risk’ group) and siblings of typically developing children with no family history of ASD (‘low-risk’ group).

In Chapter 1 a review of the existing research literature identified a broad range of potential behavioural markers of ASD, including observations of atypicalities in language, social communicative development, motor control, eye contact, visual tracking, orienting to name, imitation, social smiling, reactivity, and sensory-oriented behaviours from as early as 6-months old (Dobkins, Akshoomoff, Carver, Dorhmann, & McCleery, 2008; Veness et al., 2011; Zwaigenbaum et al., 2005). However, no single variable assessed prior to age one was predictive of later ASD outcome. This review also highlighted the limited research to date on early prenatal development in ASD, despite several preliminary studies indicating that neurobiological atypicalities associated with ASD, such as macrocephaly, may be present from birth (Gillberg & Souza, 2002; Hobbs et al., 2007). To address the gaps in this research, we recruited a prospective, longitudinal cohort study of ‘high risk’ (n = 33) and ‘low risk’ (n = 44) infant siblings called the PRegnancy Investigation of Siblings and Mothers of children with autism (PRISM) study in Perth, Western Australia. Data from this cohort were used throughout this thesis to address each of our research aims.

The first aim of this thesis was to investigate the role of prenatal brain overgrowth in ASD. Chapters 2 and 3 examined, respectively, two and three-dimensional ultrasound data collected at multiple time points during pregnancy as part of the PRISM study. Measurements of prenatal head and body growth were then compared between high- and low-risk fetuses to identify potential differences in in utero growth trajectory. Chapter 3 also investigated the association between these prenatal measures and later ASD
symptomatology. For two-dimensional ultrasounds, we observed no significant differences between the groups in measurements of head circumference and body growth. However, a novel three-dimensional ultrasound technique revealed a unique pattern of cerebral volume growth during the second trimester amongst high-risk fetuses, relative to low-risk fetuses. This difference between groups was characterised by a slow rate of growth between 17 and 21 weeks’ gestation and a more rapid rate of growth from 22 weeks onwards in high-risk fetuses. However, individuals who were diagnosed with clinical ASD at age two, did not appear to contribute to this atypical growth trajectory.

The second aim of this thesis was to determine when the behavioural differences between high- and low-risk infant siblings first emerge. We obtained a broad range of behavioural observations including infant cry acoustics, parent-child interaction, social-communication development and language ability during the first and second years of life in the high- and low-risk infants. The third aim was to examine which of these behavioural characteristics of high-risk infants, if any, are predictive of later ASD outcomes at age two. Chapter 4 compared the acoustic properties of infant cry data between 12-month old high- and low-risk infants. High-risk infants had shorter cry utterances, relative to low-risk infants. In Chapter 5, observations of parent-infant interactions obtained at 6- and 12-months old were compared between high- and low-risk infant siblings. High-risk infants presented with increased negative affect during parent-infant interaction at 6-month follow-up and a greater number of ASD symptoms at 12-months old, relative to low-risk infants. Behavioural markers at 12-months old (but not 6-months) predicted ASD outcomes at age two.

In an additional study (reported in the Appendix), we proposed a method for parsing ASD heterogeneity by investigating the behavioural phenotype associated with two possible environmental risk factors namely, maternal SSRI use and birth weight.

The results of the thesis are summarized in Chapter 6 where the broader theoretical and clinical implications of the findings are discussed. This thesis contributes data to the accumulating literature seeking to identify predictive markers for ASD in the first years of life. From the data presented in this thesis, it is apparent that differences between high-risk and low-risk siblings can be detected from as early as 6-months old and, from 12-months old, are predictive of later ASD diagnosis. In conclusion, this thesis supports the utility of examining pre- and postnatal development of high- and low-risk
siblings of ASD as a platform for understanding the disorder and highlights several future avenues for research during very early development.
References


# Table of Contents

Abstract .............................................................................................................................. i  
References ......................................................................................................................... iv  
Table of Contents ............................................................................................................. v  
List of Figures .................................................................................................................... ix  
List of Tables ..................................................................................................................... x  
Manuscripts and Publications Generated from this Thesis ........................................... xii  
Acknowledgements .......................................................................................................... xiv  
Statement of Candidate Contribution ........................................................................... xvi  

## CHAPTER 1  General Introduction: A Review of Early Risk Factors for Autism Spectrum Disorder ................................................................. 1  

- Autism Spectrum Disorder: diagnosis and prevalence ........................................... 1  
- Early intervention findings ...................................................................................... 3  
- Aetiology of ASD ...................................................................................................... 5  
- Early Risk Factors: retrospective and prospective study designs ...................... 8  
- Evidence of neurodevelopmental risk factors .................................................... 11  
- Evidence for behavioural risk factors .............................................................. 14  
- Thesis aims and organization ............................................................................. 19  
- References ............................................................................................................ 23  
- Foreword to Chapters Two and Three ............................................................... 36  

## CHAPTER 2  A Prospective Ultrasound Study of Prenatal Growth in Infant Siblings of Children with Autism ................................................... 38  

- Method ..................................................................................................................... 40  
- Participants ............................................................................................................ 40  
- Demographic information .................................................................................... 40  
- Fetal ultrasound studies ....................................................................................... 41  
- Statistical analysis ............................................................................................... 42  
- Results .................................................................................................................... 42  
- Discussion ............................................................................................................ 49  
- References ............................................................................................................ 51  

## CHAPTER 3  A preliminary study of cerebral growth in fetuses at high-risk of Autism Spectrum Disorder ................................................. 55  

- Methods .................................................................................................................... 58  
- Participants ............................................................................................................ 58
CHAPTER 4 A longitudinal study of behavioural risk markers for Autism Spectrum Disorder in high risk infant siblings

Method ......................................................................................... 84
Participants .................................................................................. 84
Demographic information .............................................................. 84
PRISM study 6-month follow-up .................................................. 85
PRISM study 12-month follow-up ................................................ 86
PRISM study two-year follow-up .................................................. 86
Statistical Analysis ........................................................................ 87
Results .......................................................................................... 87
Family demographics ..................................................................... 87
Between group comparisons at 6-month follow-up ....................... 89
Between group comparisons at 12-month follow up ...................... 90
Between group comparisons at two-year follow up ....................... 90
Relationship between early markers and two-year outcome .......... 92
Discussion .................................................................................... 94
References ................................................................................... 97

CHAPTER 5 Acoustic properties of cries in 12-month old infants at high-risk of Autism Spectrum Disorder ........................................... 109

Method ......................................................................................... 112
Participants .................................................................................. 112
Demographic information .............................................................. 112
PRISM study 12-month follow-up ................................................ 113
Acoustic analysis procedures ......................................................... 114
Coding of Cry Episodes ................................................................ 115
PRISM study two-year follow-up .................................................. 116
Statistical Analysis ...................................................................... 118
Results .......................................................................................... 118
Between-group comparisons at 12-month follow-up .................... 121
Between-group comparisons at two-year follow up ....................... 123
Relationship between early markers and two year outcome ..........125
Discussion.......................................................................................126
References.....................................................................................131

CHAPTER 6   General Discussion..................................................136
The role of prenatal brain overgrowth in ASD.................................138
When do behavioural differences between high- and low-risk infant siblings first emerge? ..........................................................140
Do behavioural characteristics of high-risk infants at 6- and 12-months old predict ASD outcomes at age two? ............................142
Implications, future directions and final conclusions .......................143
Final conclusions ............................................................................147
References.....................................................................................149

Appendix I   A 'bottom-up' approach to aetiological research in autism spectrum disorders .........................................................155
Study 1 ............................................................................................159
Materials and Methods .................................................................159
Participants ....................................................................................159
Measures and Procedure ..............................................................159
Statistical analyses .......................................................................161
Results ..........................................................................................161
Discussion .....................................................................................164
Study 2 ............................................................................................165
Materials and Methods .................................................................165
Participants ....................................................................................165
Measures and Procedure ..............................................................165
Statistical analyses .......................................................................165
Results ..........................................................................................166
Discussion .....................................................................................169
General discussion .......................................................................170
References.....................................................................................173
List of Figures

Figure 1. Rate of head circumference growth relative to femur length in high and low risk fetuses .......................................................... 49

Figure 2. Multi-level quadratic regression curves for cerebral volume growth across the second trimester. Measurements from HR fetuses are shown in green and measurements from LR fetuses are shown in blue. The black squares represent HR fetuses who received a clinical diagnosis of ASD at two years old.................................67

Figure 3. Length of phonation by risk group and ADOS-G diagnostic classification. Blue dots correspond to infants who did not receive a diagnosis of ASD and black dots correspond to infants who did receive a diagnosis of ASD.........................................125
List of Tables

Table 1. Characteristics of probands in the two groups ...........................................43
Table 2. Characteristics of the family, the pregnancy and the fetus .......................44
Table 3. Descriptive statistics showing the M (SD) gestation age in weeks, and fetal measurements (in mm) obtained at each time point ..........46
Table 4. Tests of the Effect of High- versus Low-risk Group using Linear Mixed Model Regression Analyses of HC, OFD, BPD and FL Adjusted for Gestational Age..................................................................................48
Table 5. Characteristics of probands and fetuses in the two groups. ‘High risk’ probands are siblings of fetuses in this group who are diagnosed with ASD, and ‘low risk’ probands are siblings of fetuses in this group who are typically developing. ........................................63
Table 6. Characteristics of the family, the pregnancy and the fetus .....................64
Table 7. Multi-level linear (Model 1) and quadratic (Model 2) regression models for high- and low-risk groups. ..........................................................66
Table 8. Prenatal brain measurements (cm3) of individuals who, at two years of age, had a clinical diagnosis of ASD (n = 2) or displayed ‘behavioural risk’ as measured by the CSBS DP. ......................67
Table 9. Family demographics for high- and low-risk groups. ‘High risk’ probands are siblings who are diagnosed with ASD, and ‘low risk’ probands are siblings who are typically developing. ..............88
Table 10. MACI global ratings of parent-infant interaction in HR (n = 28) and LR (n = 38) infant siblings .................................................................................90
Table 11. Characteristics of the high- and low-risk groups for categorical risk variables obtained at 6, 12 and 24-month follow up. ...............91
Table 12. Characteristics of high- and low-risk groups for continuous variables at 6, 12 and 24-month follow up .........................................................92
Table 13. Correlations between 6-month, 12-month and two-year follow up data..................................................................................................................93
Table 14. Description and biological mechanism for each of the acoustic variables ........................................................................................................117
Table 15. Characteristics of probands in the two groups. ‘High risk’ probands are siblings who are diagnosed with ASD and ‘low risk’ probands are siblings who are typically developing. .................................120
Table 16. Characteristics of the high- and low-risk infant groups ..........................122
Table 17. One-way Analysis of Variance between high- and low-risk groups for the acoustic measurements. ......Error! Bookmark not defined.
Table 18. Characteristics of high- and low-risk groups for continuous and categorical variables at 12 and 24-month follow up ...............124
Table 19. Study 1: Maternal and offspring characteristics of the SSRI case group ................................................................. 162

Table 20. Study 1: Descriptive statistics and independent-samples t-tests for CSHQ, SRS and ADOS severity scores. ......................... 163

Table 21. Study 1: Chi-square analyses using Fisher’s exact test for both groups of children for the five gastrointestinal complaints. ................. 164

Table 22. Study 2: Offspring characteristics of the LBW case group ............... 167

Table 23. Study 2: Descriptive statistics and independent-samples t-tests for CSHQ, SRS, CCC-2 and ADOS severity score. .................... 168

Table 24. Study 2: Chi-square analyses using Fisher’s exact test for both groups of children for the five gastrointestinal complaints. ................. 169
Manuscripts and Publications
Generated from this Thesis

This thesis is comprised of a collection of papers that have been prepared for submission to peer-reviewed journals. These papers are supplemented by one literature review which forms the introduction, forewords that link the experimental papers and a general discussion. The publications that have arisen from this thesis are as follows:

Chapter 2


Chapter 3


Chapter 4


Chapter 5

Appendix I

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I am thankful for my grandparents and in-laws, who have continued to tell me how proud they are of me throughout this journey. I am incredibly grateful to
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Statement of Candidate Contribution

For each of the studies that comprise this thesis, the candidate played a major role in participant recruitment and testing, data entry, analysis, and interpretation. The candidate collected data from each family who participated in the PRISM study at three time points (birth, 6-month and 12-month follow-up). Each manuscript was prepared and revised by the candidate with the assistance of her supervisors, Murray Maybery and Andrew Whitehouse. Several external authors also contributed to the manuscripts. Cheryl Dissanayake and Martha Hickey reviewed all manuscripts prior to submission and are included as co-authors for Chapter 2-5. Peter Jacoby and Patrick Dunlop collaborated with the candidate to run statistical analyses for Chapter 2 and 3, respectively. Anthony Murphy, Wendy Lilje, Michelle Bellesini and Craig Pennell (ultrasonographers) performed all of the ultrasound scans reported in Chapters 2 and 3. Anna Hunt and Joanna Granich (research assistants) were instrumental in participant recruitment and are included as co-authors for Chapters 2, 3 and Appendix I. Victoria Reynolds, Ildi Bruz and Natalie Ciccone collaborated with the candidate to perform the acoustic analyses in Chapter 4. Ming Wai Wan and Ami Brooks coded video observations of parent-infant interaction and are included as co-authors for Chapter 5.

The study presented in Appendix I used data that was collected as part of the Western Australian Autism Biological Registry (WAABR), an ongoing study of families of children with autism. WAABR is co-ordinated by one of the candidate’s supervisors, Andrew Whitehouse. John Wray was instrumental in establishing WAABR and Anna Hunt oversees the daily management of the registry and have all been included as authors for Appendix I.
Each author has given permission for all work to be included in this thesis.

Lisa Unwin (candidate) Date

Murray Maybery (coordinating supervisor) Date

Andrew Whitehouse (supervisor) Date

**Autism Spectrum Disorder: diagnosis and prevalence**

Autism Spectrum Disorder (ASD) is characterised by two symptom domains: (1) impairments in social-communication, and (2) the presence of repetitive interests and behaviours (*Diagnostic and Statistical Manual for Mental Disorders* (DSM-5); American Psychiatric Association, 2013). Behaviours described under these two domains include: limited social and emotional reciprocity, deficits in nonverbal communication, use of stereotyped movements and/or speech, and ritualised patterns of behaviour. The DSM-5 has introduced severity criteria to better capture the spectrum nature of the disorder. These criteria are used to explain the intensity and duration of symptoms, the degree of impairment, and the level of distress. Although the severity of ASD can vary, the impairments in social-communication may result in significant effects on a child’s development, severely compromising the quality of daily living into adulthood (Howlin, Goode, Hutton, & Rutter, 2004). ASD is associated with longer-term consequences on educational and vocational attainment (Whitehouse et al., 2009a; Whitehouse et al., 2009b) and results in high economic costs for families and the community (Horlin, Falkmer, Parsons, Albrecht, & Falkmer, 2014). While the social, emotional and lifestyle strain on affected individuals and their families are challenging to quantify, the annual public health burden of ASD in Australia alone is estimated to be between $4.5 and $7.2 billion (Synergies Economic Consulting, 2008).

Prevalence estimates for ASD have increased dramatically over the past 40 years (Fombonne, 2003). Early epidemiological studies estimated the prevalence of ASD to be around 4 cases per 10,000 (Lotter, 1966), compared with more recent reports that estimate
ASD currently affects 1% of the general population (Baird et al., 2006) or 1 in 68 children (Baio, 2012). The extent to which the reported increase in prevalence reflects a true increase in the disorder, rather than better use of diagnostic tools and greater awareness of the disorder, has been questioned (Fombonne, 2003).

The incidence of ASD diagnoses are higher in males compared to females, with prevalence estimates indicating that there is a 4:1 diagnostic bias towards males (Werling & Geschwind, 2013). It has been proposed that this male bias may be a result of current diagnostic practices, whereby clinicians are more likely to diagnose males with ASD than females, even when both sexes have symptoms associated with the disorder documented in educational and clinical records (Giarelli et al., 2010). However, research has also supported the role of biology in this sex difference in ASD, whereby exposure to fetal androgens (e.g. testosterone) during critical periods of development has been found to influence structural and functional brain development in areas that are known to be associated with ASD (Baron-Cohen et al., 2011; Lombardo et al., 2012). Genetic factors have also been implicated in the male preponderance in ASD. Whereby, the recent ‘female protective hypothesis’ proposes that the presence of genetic variations unique to females protects them from neurodevelopmental disorders such as ASD, and thus an increased aetiological burden is required to manifest the same severity of impairment as compared to males (Jacquemont et al., 2014; Robinson et al., 2013).

Currently, ASD cannot be diagnosed reliably until the second year of life (Elsabbagh & Johnson, 2010), despite evidence supporting the presence of ASD behavioural symptoms in infants as early as 6-months old (Barbaro & Dissanayake, 2009; Yirmiya & Charman, 2010) and unique neurodevelopmental characteristics during prenatal development (Gillberg & De Souza, 2002; Hobbs et al., 2007; Stoner et al., 2014). These observations consistently support the early emergence of ASD, and have directed researchers to investigate methods for identifying risk factors, as well as exploring the impacts of early diagnosis on the ASD developmental trajectory.

In Western Australia, diagnoses of ASD require agreement from a paediatrician, speech pathologist and psychologist (Glasson et al., 2008). This typically involves rigorous
assessment using the *Autism Diagnostic Observation Schedule* (ADOS; Lord et al., 2000) and/or *Autism Diagnostic Interview-Revised* (Rutter, Couteur, & Lord, 2003) in combination with observation and clinical judgement. However, our current diagnostic tools cannot reliably diagnose children prior to age two. Since we now have well-established research supporting the early emergence of ASD, clinicians and researchers have directed their attention toward identifying infants who are at high-risk of a later diagnosis. Teams of developmental researchers worldwide are now dedicated to identifying a range of risk factors for ASD that may be seen in children, prior to age two. Early identification of infants at risk for disorder, during critical developmental stages and prior to an onset of the core ASD symptoms, may have the potential to influence the symptom trajectory and reduce the long-term impact of disorder (Webb, Jones, Kelly, & Dawson, 2014).

### Early intervention findings

The first interventions designed to target the early symptoms of ASD were based primarily on psychological learning theory. Early Intensive Behavioural Intervention (EIBI), such as applied behaviour analysis, involves multiple clinicians working with the child for 30-40 hours per week, typically over 2-4 years (McEachin, Smith, & Ivar Lovaas, 1993). In several randomized-controlled trials (RCTs) comparing EIBI with treatment in the community, EIBI has seen some benefits in improving children’s general cognitive ratings but no superiority, relative to community treatment for improving adaptive functioning or autism-specific behaviours (Howlin et al., 2009; Roberts & Prior, 2006; Smith, Groen, & Wynn, 2000).

Dawson et al. (2010) reported on the first randomized-controlled trial (RCT) of the Early Start Denver Model (ESDM) for pre-school-aged children with ASD. ESDM is a comprehensive developmental intervention that expands the behavioural approach of EIBI to work with parents to develop skills in social reciprocity. This RCT (n = 48), compared ESDM (consisting of an average of 15 hours per week clinician input, and 16 hours per week parent-guided intervention) over two years, with treatment as usual in the general community. Children who received the ESDM intervention demonstrated relative
improvements in language, IQ scores and adaptive functioning, relative to children who received treatment as usual. However, ESDM had no effect on other ASD-specific symptoms. In a follow-up investigation, Dawson et al. (2012) studied the effect of ESDM on brain activation patterns by measuring electroencephalogram (EEG) activity. Typically developing children and children receiving ESDM showed increased cortical activation and a shorter Nc latency when viewing faces, relative to the treatment as usual group. This evidence suggested that abnormal brain circuitry in ASD precedes atypical social behaviours, and that interventions designed to promote early social skills may have the potential to change the trajectory of ASD (Webb et al., 2014).

A further study by Dawson et al. (2012) investigated the efficacy of a parent-guided version of the Early Start Denver Model (P-ESDM) relative to treatment as usual in the general community. This RCT involved 98 toddlers aged 14-24 months identified as at-risk of ASD via referrals from trained clinicians. Dawson et al. (2012) reported that a greater number of intervention hours, as well as a younger age at the start of intervention, were associated with improvements in ASD-related behaviours for children who received P-ESDM. However, there were no significant improvements in parent-child synchrony for either the P-ESDM or treatment as usual groups. To date, parent-guided interventions for ASD are yet to show the large effects seen in intensive intervention trials (Dawson et al., 2012).

Since diagnostic age of ASD is relatively late, one critical step in applying early intervention is to be able to identify children at-risk of disorder prior to receiving a formal diagnosis. Interventions designed to target early markers of ASD can then capitalise on neural plasticity very early in development, maximising the benefits of therapy (Dawson, 2008). Green et al. (2015) recently conducted an RCT examining the effect of a parent-mediated intervention for 7-10 month-old infants at risk of ASD. This intervention was designed to target disruptions to the parent-child interaction that are thought to influence ASD and the social-developmental trajectory. Immediately post-intervention, infants receiving the treatment were found (relative to the control group) to have increased attentiveness to their parent, reduced ASD-risk behaviours, increased parental non-directiveness towards their child, improved attention disengagement and improved parent-
rated adaptive functioning (Green et al., 2015). These observations highlight the potential for early parent-child interaction markers to inform treatment and reduce the impairment associated with the disorder.

This brief review of the literature highlights that there are great benefits of administering early intervention programs with children at-risk of ASD. Interventions that occur during the “pre-symptomatic period” for ASD may capitalise on neuroplasticity and reduce the manifestation of the core or secondary symptoms, thus altering the ASD trajectory (Dawson, 2008; Lewis, 2004). However, in order to identify infants who are pre-symptomatic, further investigation of potential risk markers for ASD is required. The following section will review what is currently known about the aetiology of ASD and what still remains inconclusive.

**Aetiology of ASD**

To date, there has been no evidence to support a single genetic or environmental risk factor that is causative in all individuals with an ASD diagnosis. The aetiological basis of ASD continues to be researched worldwide with a great focus on genetics and the gene-environment interaction (i.e. epigenetics), with a particular emphasis on the pre-conception, conception and pregnancy environment. Due to the heterogeneity of ASD, it has been proposed that ASD has multiple aetiological risk factors that differ across individuals (Whitehouse & Stanley, 2013). With the potential for tens, or perhaps hundreds of aetiological pathways to ASD (Betancur, 2011), it becomes even more challenging for researchers and clinicians to identify which children are at risk of disorder.

**Genetics.** Behavioural and molecular genetic research has shown that genetic factors play a substantial role in the aetiology of ASD. Studies have observed a concordance rate of between 77% and 92% for ASD in monozygotic twins, compared with 10-31% in dizygotic twins (e.g. Bailey et al., 1995; Hallmayer et al., 2011) and have reported estimates of an 18-20% recurrence rate of ASD among siblings (Ozonoff et al., 2011). The heterogeneous clinical presentations among individuals with ASD has hindered researchers in search for a common genetic profile. The presence of ASD susceptibility loci
has been implicated in several chromosomal regions in genetic studies, however, there has been no replication or consistent evidence to support the association between any common genetic variants and an increased risk of ASD (Hagerman & Hendren, 2014).

There are recent estimates of over 500 genetic loci that may be implicated in ASD (Stessman, Bernier, & Eichler, 2014). Genetics investigations have detected de novo variations (such as duplications at 15q11-13) and genetic mutations (e.g. CHD8, SNC2A and POG2) that are strongly linked with the ASD behavioural phenotype (State & Levitt, 2011; Murdoch & State, 2013). Previous studies have also identified rare mutations in genes associated with synaptic function (e.g. SHANK3, SHANK2 and NRXN1) in ASD, which have also been linked to other disorders with genetic origins (State & Levitt, 2011). Finally, the overexpression of rare, de novo structural variations have been detected in the genome of simplex families (one affected child), relative to multiplex families and families with no history of ASD (State & Levitt, 2011).

Researchers continue to investigate this complex heterogeneous genetic aetiology and have homed in on disorders like Fragile X that show comorbidities with ASD, and thus similar behavioural manifestations in order to further understand the aetiological pathways to ASD. Whilst important, genetic susceptibility does not explain everything. Environmental exposures and gene-environment interactions are involved in all stages from pre-conception through to early postnatal development.

**Environment and epigenetics.** The less than 100% concordance for ASD amongst monozygotic twins (e.g. Bailey et al., 1995; Hallmayer et al., 2011), the variability in symptoms, and degree of impairment within ASD-concordant twins, implicates the role of environment and epigenetic factors in the aetiology of the condition (Wong et al., 2014). The environment is inclusive of all non-genetic exposures such as viruses, chemicals, medications, as well as social-cultural influences (Lyall, Schmidt, & Hertz-Picciotto, 2014). Exposure to environmental factors may affect brain development at different stages, including cell differentiation and migration, synaptogenesis, the formation of the neural tube and cortical mini-columns (e.g. Lyall et al., 2014). Oxidative stress (i.e. immune system dysregulation) and deficiencies in essential fatty acids or nutrients have also been
implicated in ASD (Lyall et al., 2014). In addition, environmental exposures may interact with susceptibility genes leading to changes in gene expression and ASD trajectory. Further, genes may alter the biochemistry of the brain via their influence on receptors, metabolism and activity of xenobiotic chemicals (Lyall et al., 2014). Evidence of the association between ASD risk and de novo alterations to DNA not inherited from parents, indicates that environmental exposures may cause damage to the genetic code (Lyall et al., 2014).

A recent literature review of environmental toxins revealed that Phthalates, Pesticides, polychlorinated biphenyls, solvents, heavy metals, air pollutants and toxic waste sites have all been implicated in ASD (Rossignol, Genuis, & Frye, 2014). Other research has observed high levels of other toxins, including aluminum, cadmium, lead, mercury and arsenic in individuals with ASD (Yasuda, Yasuda, & Tsutsui, 2013). Recent research has also supported the impact of maternal use of prescription medications such as valproic acid (Moore & Calvert, 2000; Rasalam et al., 2005) and thalidomide (Gillberg, 1999; London, 2000; Strömland et al., 1994) on increased ASD risk. To date, evidence for the association between maternal smoking and alcohol use with ASD is weak, and requires further investigation (Lyall et al., 2014). Whereas evidence of the association between obstetric risk factors such as caesarian delivery, low birth-weight, premature birth, and uterine bleeding with ASD have been more consistent (Croen et al., 2005; Glasson et al., 2008; Hultman, Sparén, & Cnattingius, 2002; Larsson et al., 2005). However, the underlying mechanism of these obstetric factors that contribute to ASD is largely unknown and the timing between the obstetric event and the actual biological onset of ASD remains unclear (Newschaffer et al., 2007).

Despite the effects of these pre-existing vulnerabilities or subsequent exposure to harmful toxins, there is some evidence to suggest that the impact of these environmental factors can be modified. For example, elevated maternal use of folic acid supplements during periconception has been linked to a reduction in ASD risk (Schmidt et al., 2012). There is also some preliminary evidence supporting the association between maternal use of prenatal vitamins and essential fatty acids with a reduction in ASD risk, (e.g. Schmidt et al., 2012; Surén et al., 2013) but this research requires replication and extension.
This brief review of environmental and genetic research in ASD highlights the complexities of identifying risk factors for disorder. Potential biomarkers with the strongest evidence include those for oxidative stress, immune function, and mitochondrial function (Lyall et al., 2014). While there is promising evidence supporting the use of biomarkers to guide intervention and identify individuals who may be at-risk of ASD, there is not currently enough evidence to support their routine clinical use (Lyall et al., 2014).

The importance of methodology. Previous research in ASD has often adopted a ‘top-down approach’ which involves constraining behavioural phenotypes of disorder in an attempt to facilitate the identification of common biological subtypes (e.g. Buxbaum et al., 2001). However, this approach has often failed to yield a more genetically homogenous sample (Geschwind & Levitt, 2007). Few studies have employed a ‘bottom-up’ approach as a means of identifying biological subtypes of ASD. This methodology focuses on known aetiological risk factors for ASD (e.g. obstetric factors; Croen et al., 2005), and investigates whether individuals exposed to these risk factors have a more homogenous phenotype (Unwin et al., 2013). Through analysis of a database of children with ASD in Western Australia, we have provided preliminary evidence of the applicability of this ‘bottom-up’ methodology in Appendix I.

Top-down and bottom-up approaches are well suited to populations of individuals who have already received a diagnosis of ASD. In order to identify potential risk factors prior to diagnosis - in the hope that clinicians can find an opportunity to provide early intervention - prospective and retrospective research designs have been employed to identify children and infants considered at-risk for disorder. The following sections will expand on our current understanding of at-risk infants and children using retrospective and prospective study designs.

Early Risk Factors: retrospective and prospective study designs

Children are typically diagnosed with ASD around the age of three years (Bent, Dissanayake, & Barbaro, 2015). However, symptoms of ASD have been observed as early as 6-12 months old (Szatmari et al., 2016). There is a large body of research that suggests
there may be profound long-term benefits for some children who obtain an early diagnosis and subsequent intervention for ASD. Early infant development has been a focus of ASD research with the aim of addressing the gap between the time a child first shows signs of ASD, and the time they receive a diagnosis. Developmental research, particularly studies that employ longitudinal designs, have the added benefit of providing insight into the trajectory of ASD symptoms during early infancy. Identifying early markers or behaviours of ASD during infancy, may have the potential to further inform appropriate targets and strategies for early ASD intervention.

Previous studies have examined retrospective parent-report and video footage of children diagnosed with ASD to inform our current understanding of early symptomatology. Retrospective studies are valuable but are limited by either the selectivity of video-recorded events (i.e. first birthday) and/or by parents’ memory of the event (Zwaigenbaum et al., 2009). Another methodology for examining the symptom trajectory of ASD prior to a diagnosis is to investigate siblings of children with ASD. Siblings of ASD probands have an estimated 18.7% increased risk for disorder themselves (Ozonoff et al., 2011), and since biological siblings share approximately 50% of their segregating genes, it is more likely that irrespective of whether the sibling is later diagnosed with ASD, they will display some of the characteristics associated with the ASD behavioural phenotype (Ozonoff et al., 2011). This prospective design involves following a ‘high-risk’ group comprised of the younger siblings of children with ASD and a ‘low-risk’ comparison group, comprised of children who have no family history of ASD (e.g. Zwaigenbaum et al., 2009).

Prospectively monitoring infant development enables researchers to observe standardized samples of behaviour and incorporate an extensive range of tools such as neuroimaging, eye-tracking, and voice analysis (Ozonoff et al., 2011). Sibling studies tend to collect data longitudinally from early infancy up until 2-3 years of age, at which point a diagnosis can be made. The prospective method facilitates inferences about possible underlying mechanisms of ASD that develop as early as the prenatal period. Findings from prospective studies may lead to the identification of novel biomarkers indicative of ASD risk; new screening measures for early symptoms of ASD (e.g. Haglund et al., 2016); and
updated models of clinical pathways for identification of risk and diagnosis of ASD (Whitehouse et al., 2017).

To date, few behavioural markers of ASD have been identified prior to 12-months of age, with greater evidence for observable symptomatology emerging during the second year. These early symptoms include repetitive and sensory behaviours and deficits in social-communication (Jones et al., 2014). During this early period of development, infants are learning how to process their external world and regulate their internal physiological state. Research indicates that in the first year of life, infants who are later diagnosed with ASD are processing this information in a way that distinguish them from typically developing infants (e.g. Elsabbagh et al., 2015; Shic, Macari & Chawarska, 2014; Wagner et al., 2016). For example, studies employing eye-tracking techniques have observed a different pattern of social attention in HR-ASD infants relative to LR and HR-no ASD infants at 6-months old (e.g. Wagner et al., 2016). These differences in social attention and gaze patterns have been linked to functional alterations in the neural circuitry in the cerebrum of individuals later diagnosed with ASD, particularly in areas such as the fusiform gyrus, inferior occipital gyrus and amygdala in children with ASD (Adolphs, Sears & Piven, 2001; Domes et al., 2013). Children with ASD have also been shown to have an enhanced response to touch in the primary somatosensory cortex (Kaiser et al., 2015). Such differences in how these infants’ process external sensory stimuli can in turn influence how they respond and interact with their environment, and further still, how primary caregivers then respond to their infant. Theoretically, it is this interactional pattern between infants showing early signs of ASD and their primary caregivers that can be interrupted and re-directed towards a typical trajectory via early intervention (Dawson, 2008; Elsabbagh & Johnson, 2010; Green et al., 2015).

Altered neural connectivity may explain unique neurodevelopment and symptomatology that is characteristic of ASD. Research in the past decade has pointed towards a theory that the brains of individuals who are later diagnosed with ASD are differentiated from typical development by “local over-connectivity” during the first years of life (Courchesne & Pierce, 2005). Specifically, individuals with ASD have an abundance of local, short-range neural connections but are deficient in long-range connections.
This abundance of local and deficit in long-range connections is positively correlated with severity of ASD symptoms (Dajani & Uddin, 2015). The unique neural connectivity networks in the brain have huge implications for how individuals with ASD receive, process and store information from their environment (Dajani & Uddin, 2015). Brain overgrowth is one such marker of this unique neurodevelopmental in ASD. Observations from post-mortem studies indicate that early brain overgrowth may be caused by an acceleration in growth of cortical gray matter which comprises development and refinement of cortical white matter (Courchesne & Pierce, 2005; Ha et al., 2015). Atypical neural connectivity may be a promising biomarker for ASD (Maximo et al., 2014) and brain overgrowth can be one clinical indicator of risk for disorder.

To summarize, identifying risk markers for ASD has the potential to contribute to earlier diagnosis and inform intervention. This research is also incredibly valuable to our current understanding of when this disorder first emerges, and the biological mechanisms that are driving the neurodevelopmental differences and subsequent behaviours that are characteristic of individuals with ASD. The most reliable evidence for neurodevelopmental and behavioural risk factors are reviewed in the following sections.

**Evidence of neurodevelopmental risk factors**

Biological risk factors for an ASD diagnosis, or ‘biomarkers’, have been the focus of considerable research attention. Brain overgrowth (measured by head circumference), exposure to prenatal testosterone and environmental toxins are just a few of the potential candidates with some supporting evidence. However, no single distinguishing neuropathologic feature has been identified, and no single model of atypical neurodevelopmental is widely accepted (Newschaffer, Fallin, & Lee, 2002).

Macrocephaly was one of the phenotypic features of ASD first described by Kanner (1943) and is now one of the most well-replicated observations in the study of biomarkers in ASD (Dissanayake, Bui, Huggins, & Loesch, 2006; Redcay & Courchesne, 2005). Head
circumference is positively correlated with brain size in infancy (Bartholomeusz, Courchesne, & Karns, 2002), and it is hypothesised that accelerated head circumference growth reflects an abnormal brain volume in children with ASD (Hazlett et al., 2017; Redcay & Courchesne, 2005). Meta-analyses have reported a rapid period of postnatal head circumference growth in ASD, such that brain volume is greater than the population average in an estimated 90% of children who are later diagnosed with the disorder (Courchesne, Campbell, & Solso, 2011). In perhaps the most comprehensive study of head circumference to date, Courchesne, Carper, & Akshoomoff (2003) examined 43 infants later diagnosed with ASD, finding that an increased acceleration in occipitofrontal head circumference growth between birth and 6-14 months was associated with greater symptom severity at 2-5 years as measured by the ADOS-G (Lord et al., 2000). Hazlett et al. (2017) recently observed further support for this phenomenon whereby they performed MRI scans with 106 HR and 42 LR infants. Observations of significant overgrowth of cortical surface area were reported for the HR infants during 6-12-months of age, relative to the LR infants, followed by a period of brain volume overgrowth (between 12-24months) in 15 of the 106 HR infants, who were later diagnosed with ASD. Furthermore, Hazlett et al. (2017) linked this period of brain overgrowth to the time of ASD symptom onset and the severity of symptoms.

The observation of increased head circumference and brain volume growth in early childhood has been replicated across some (Gillberg & De Souza, 2002; Redcay & Courchesne, 2005; Hazlett et al., 2017), but not all studies (Raznahan et al., 2013; Shen et al., 2013). Approximately 20% of children with ASD are estimated to have a head circumference measuring at two or more standard deviations above the mean for their age group (Miles, Hadden, Takahashi, & Hillman, 2000). Importantly, macrocephaly has been observed in newborns who are later diagnosed with ASD at twice the prevalence of the general population (Gillberg & Souza, 2002; Redcay & Courchesne, 2005), indicating that for some individuals with ASD, atypical neurodevelopment may commence prenatally. However, for others, children with macrocephaly at the time of diagnosis tended to have an average head size at birth (Gillberg & Souza, 2002; Lainhart, 2006). Again, this research highlights the heterogeneity in symptomatology amongst individuals diagnosed with ASD.
In addition to overall brain growth, subregions of the brain have been linked to the manifestation of ASD symptomatology. One brain region often found to be abnormal in ASD is the cerebellum (Fatemi et al., 2012). Postmortem brain studies have reported that regardless of sex, cognitive ability and age, individuals with ASD have fewer Purkinje cells in the cerebellar hemispheres, compared to typically developing individuals (Arin, Bauman, & Kemper, 1991; Bailey et al., 1998; Bauman & Kemper, 1994; Whitney, Kemper, Bauman, Rosene, & Blatt, 2008). Furthermore, one particular study (Sparks et al., 2002) using magnetic resonance imaging (MRI) reported enlarged cerebellum volume in children with ASD, relative to typically developing children.

Many cortical and subcortical structures connect with the cerebellum to modulate language, cognition, motor, emotional and sensory functions (Schmahmann, 1997). The cerebellum is also involved in the production of mental imagery, aspects of attention, anticipatory planning, affective behaviour, and visual spatial organisation (Fatemi et al., 2012). Studies using structural and functional MRI have found that these important cognitive functions may be disrupted in ASD and that the reported cerebellar abnormalities in the brains of individuals with ASD may potentially contribute to these core symptoms of the disorder (Fatemi et al., 2012).

The amygdala is another subregion of the brain that has been implicated in the manifestation of ASD symptomatology. It has been hypothesised that abnormal amygdala volume is related to some of the core social characteristics of ASD (Baron-Cohen, Ring, & Bullmore, 2000; Mosconi et al., 2009). Postmortem studies of individuals with ASD have noted immature-appearing and densely packed cells (Bailey et al., 1998; Bauman & Kemper, 1994) and fewer neurons within the amygdala (Schumann & Amaral, 2006). Additionally, Mosconi et al. (2009) found a significant relationship between amygdala volume and joint attention, which indicates that alterations to this brain region may be linked to clinical features of ASD.

**Does brain overgrowth begin prenatally?** Several key findings have led researchers to hypothesize that the process of brain overgrowth in individuals with ASD may begin prenatally. In 2007, Hobbs et al. obtained retrospective fetal ultrasound scans of
45 children with ASD and 222 children who had developed typically. The children who later received a diagnosis of ASD did not differ significantly from typically developing children on measurements of head circumference, femur length and abdominal circumference recorded during the second trimester (at a mean of 19 weeks gestation). However, the mean biparietal diameter relative to head circumference (used as an index of head shape), was observed to be significantly increased in the children with ASD, providing preliminary evidence of a ‘subtle disturbance in the uniformity’ of fetal brain growth (Hobbs et al., 2007). Whitehouse et al. (2011) also conducted a large prospective study of prenatal head circumference growth using ultrasound data from a general population pregnancy cohort. Of the study sample, second trimester ultrasound data were obtained for 14 children with ASD, who were then each matched with four typically developing control children (N =56). This study observed no significant differences between groups for head circumference size at either of the two time-points (18 weeks gestation and birth). However, four of the children with ASD (36.4%) were considered to have an enlarged head circumference measurement relative to their body size (determined by a predefined threshold), compared to 0% of the control group. More recently, Stoner et al. (2014) obtained post-mortem neocortical tissue samples from children aged 2-15 years and analysed potential markers for glia, neurons and genes that have been associated with ASD. Atypical cortical cytoarchitecture and disorganization of neurons was observed in the prefrontal and temporal cortical tissue from 90% of children diagnosed with ASD, compared with 10% of typically developing children. These previous studies have been somewhat limited by small sample sizes and retrospective study designs that do not facilitate follow-up of individuals with ASD. Further investigation is required in order to elucidate the critical periods of prenatal development where head enlargement begins (Unwin et al., 2016).

Evidence for behavioural risk factors

In addition to the study of neurodevelopmental markers, retrospective parent-report and home-videos have consistently identified behavioural differences among infants who are later diagnosed with ASD, relative to infants who develop typically (Jones et al., 2014). The emergence of early behavioural symptoms of ASD may precipitate changes in the
child’s attention towards their environment, which in turn limits the opportunity for skill advancement (Jones et al., 2014). The degree of impairment may also be determined by learnt compensatory skills, pre-existing vulnerabilities and protective factors (Jones et al., 2014). Understanding the aetiology and the very early trajectory of ASD is critical for identifying children who are at-risk of ASD and for designing appropriate interventions (Jones et al., 2014).

**Social interaction.** At 6-months of age, the ability to engage in social interactions tends to be comparable between infants who are later diagnosed with ASD, and infants who develop typically. For example, no significant differences in the use of social skills such as gaze, affect, vocalization and smiling have been reported in at-risk infants relative to typically developing infants (Rozga et al., 2011; Young et al., 2009). Although studies suggest social interaction may be relatively intact at 6 months, by 12-months old, the ability to initiate joint attention and integrate social gestures (e.g. pointing or showing items for interest to others) tends to be delayed in infants later diagnosed with ASD, compared to typically developing infants (Barbaro & Dissanayake, 2013; Landa et al., 2007; Macari et al., 2012). Contrary to these observations, Rozga et al. (2011) reported no such difference in the use of gaze to initiate joint attention in at-risk infants. Thus, there are some inconsistencies within the literature and it also remains unclear whether reduced gaze alternation is unique to infants later diagnosed with ASD, or whether it is also common to children who are language and/or developmental delayed.

**Response to social interaction.** From a few hours after birth, typically developing infants indicate a preference for faces over objects (e.g., shapes), and show a fondness for their mother’s face over a stranger’s face (e.g. Bushnell, 2001; Johnson, Posner, & Rothbart, 1991). This early interest in faces marks the beginning of social skill development. At 6-months, social attention to a stranger appears typical in infants later diagnosed with ASD (Ozonoff et al., 2010). In a recent study, Elsabbagh, Fernandes, Webb, & Dawson (2013) used an eye-tracking device and a computerized task displaying a static face amongst non-social distractors, and found no differences between high- and low-risk infants in orienting or visual attention at 6- and 12- months old. Yet, Chawarska, Macari, & Shic (2013) found that 6-month-old infants who are later diagnosed with ASD, directed
their attention to the face of an adult experimenter and to the screen less frequently than infants who developed typically. Again, there are inconsistencies in findings from research of infants prior to 12-months, which may reflect differences in the methodology (e.g. naturalistic vs. clinic setting and prospective versus retrospective designs). Further investigation is required for the early postnatal months to help clarify these findings.

Several studies have identified clear deficits in social attention in infants at-risk of ASD. At 12-months old, infants who are later diagnosed with ASD show decreased preference for faces and direct less vocalizations towards others than typically developing infants (Ozonoff et al., 2010). Among infants aged 6-24 months, the frequency of social smiling and gaze towards faces progressively declined in the infants who were later diagnosed with ASD, and directed vocalizations did not increase at the same rate as typically developing infants (Ozonoff et al., 2010). Deficits in responding to own name tended to emerge between 9-12 months in infants later diagnosed with ASD (Feldman et al., 2012; Nadig et al., 2007). Naturalistic observation studies of mother-infant interaction have demonstrated that infants diagnosed with ASD at age three, showed reduced attentiveness to their mother and reduced dyadic mutuality at 12- but not 6-months of age, relative to high- and low-risk infants without a diagnosis of ASD (Wan et al., 2012, 2013). Clifford et al. (2013) also reported that parents of infants who are later diagnosed with ASD rated their infants as less affectionate toward them and less likely to smile during a playful interaction at 14-24 months, but not at 7-months of age, relative to low-risk infants.

Deficits in imitation have also been observed in infants at-risk of ASD (Rogers, Hepburn, Stackhouse, & Wehner, 2003) and are associated with language ability (Toth, Munson, Meltzoff, & Dawson, 2006). High-risk infant siblings who are later diagnosed with ASD show reduced imitation of sounds or words at 12-18 months, relative to high- and low-risk infants who develop typically (Feldman et al., 2012). Observations of functional and symbolic imitation at 12-months old also distinguished high- and low-risk infants who met criteria for ASD, from those who developed typically (Macari et al., 2012). Comprehensive longitudinal investigations of behavioural risk markers in high-risk samples are required to better elucidate the influence of imitation deficits on the developmental trajectory of ASD (Jones et al., 2014).
Language and communication. Impairments in non-verbal and verbal communication are key behavioural features of ASD. Recent evidence suggests that one of the first modes of infant communication, crying, may be atypical in infants who are later diagnosed with ASD. Sheinkopf, Iverson, Rinaldi, & Lester (2012) recently reported that 6-month-old high-risk infants who were later diagnosed with ASD recorded cries that were poorly phonated compared to cries of infants who develop typically. Delays in expressive language have also been observed in the first year of life in high-risk infants relative to low-risk infants, with atypicalities in babbling and delayed production of first words (Iverson & Wozniak, 2007). Receptive language delays have been reported for high-risk infants, whereby, at 12-months, high-risk infants show lower scores on developmental measures such as the Mullen Scales for Early Learning (MSEL; Mullen, 1995) that assess their ability to understand phrases such as “give it to me”, “give it to mummy” and “no!”, relative to low-risk infants (Zwaigenbaum et al., 2005).

Again, it is important to note that not all studies have observed delays in expressive and receptive language amongst infants later diagnosed with ASD. For example, Hudry et al. (2014) found that infants later diagnosed with ASD showed poor expressive language, relative to those who developed typically, but no differences in receptive language were observed at any age. Similarly, Talbott et al. (2015) reported intact expressive and receptive language on the MSEL for nine 18-month-old infants who later received a diagnosis of ASD. The absence of a delay in language in some children with ASD and the strong language skills of the high-risk ASD group at follow-up, suggest that there is variability in language development within ASD. Therefore, measuring language ability at a young age, could serve as a good predictor for later functioning level (Jones et al., 2014).

This review of the literature indicates that there are several behavioural markers that may facilitate the early identification of ASD. The differences identified in infants at risk of ASD also provide possible targets for intervention. For example, based on observations made by Wan et al. (2012) of reduced attentiveness and reduced dyadic mutuality of infants towards their caregivers during play, Green et al. (2015) are conducting a large RCT to investigate the efficacy of a video-based intervention targeting parent-infant synchrony. Research for early risk markers for ASD is critical to identifying targets for early
intervention. The role of interactive synchrony in altering the developmental trajectory of at-risk infants and reducing impairments is one promising avenue for further research.

A review of the literature highlights the current inconsistencies among research of infants at-risk of ASD aged between 0-12-months old. There are several factors that appear to hinder the search for a common risk marker for ASD in very early postnatal development. The first factor relates to the data we have presented in Appendix I of this doctoral thesis. Research continues to provide evidence that the spectrum of autism symptoms is broader than first thought, and that a ‘bottom up’ methodology when researching ASD could be a more lucrative approach moving forward. For example, grouping infants primarily on their ‘high’ or ‘low’ risk status may still introduce great variability and limit the opportunities of finding risk markers that are common to all HR infants who are later diagnosed with ASD. During this early stage of development, it can also be very difficult to differentiate those infants that are HR of ASD from those who are presenting with language or social-skill delays, and thus, without following these infants to later diagnostic age, the findings from these studies have limited clinical utility.

Another factor that may contributed to the limited findings in ASD-risk research relates to small sample size. The HR/LR longitudinal study design often requires ongoing participation and follow-up from families who are already required to attend regular appointments for their child with ASD. It can also be challenging to recruit pregnant mothers who have an existing child with ASD. Research indicates that upon learning that their child has been diagnosed with ASD, parents choose not to have more children (reproductive stoppage; Hoffmann et al., 2014; Wood et al., 2014). In small studies, statistical power is often too low to detect a significant effect, which may explain some of the inconsistencies between studies. Further, small numbers of participants make it more difficult to reliably interpret the data and limit the clinical usefulness of identified risk markers.

A final factor that may influence research for reliable risk markers for ASD relates to the challenges of assessing infants in early development. Often some of the behavioural nuances that are being assessed (e.g. eye-contact, temperament, reciprocal engagement) are
influenced by whether the infants are fatigued, fed and changed by caregivers during the assessment, and even whether they are distracted by the presence of other siblings. Whilst this is a general challenge associated with all infant research, it may explain some of the inconsistencies within research that aims to measure and quantify subtle differences in social behaviours.

This thesis adopts a HR/LR longitudinal design in a relatively small sample, and thus cannot address all of these potential factors that contribute to inconsistencies in the current body of research. However, this thesis has employed novel technology and comprehensive assessment of infant development, administered by trained clinicians to better and perhaps more objectively, quantify risk markers of ASD. This thesis also includes two studies that aim to provide some replication and advancement of previously researched risk markers (e.g. cry and social behaviours), with the aim of providing further evidence to support their clinical utility in identifying infants at-risk of ASD. The following section provides a break-down of the thesis organisation and aims. A summary of the studies’ methodology and participants are included in Table 1.

**Thesis aims and organization**

A review of the empirical literature indicates that the prenatal period of development has been largely unexplored in relation to risk markers for ASD. It is also apparent that many of the postnatal behavioural factors that have been implicated in ASD require further replication. The data for this thesis were collected as part of the PRegnancy Investigation of Siblings and Mothers (PRISM) of children with autism study, Perth Western Australia. The PRISM study is comprised of two groups of pregnant mothers: 1) mothers with an existing child who has been diagnosed with ASD (‘high risk’) or 2) mothers with existing children with no family history of ASD (‘low-risk’). These mothers were recruited in the early stages of pregnancy and were invited, along with their families, to participate in this three-year longitudinal study. Their participation in the study involved three prenatal ultrasound scans, collection of maternal blood samples at 18 weeks gestation and samples of their newborn’s umbilical cord blood. The mothers also agreed to
participate in three postnatal follow-up appointments when their infant was 6, 12, and 24 postnatal months, as well as completing additional assessment measures when their infant was 9- and 18-months old.

The first aim of this thesis was to provide further data on neurodevelopment in prenatal life in order to explore the brain overgrowth hypothesis in ASD research. These observations are presented in Chapters 2 and 3. The second aim was to determine when the behavioural differences between high- and low-risk infant siblings first emerge. We obtained a broad range of behavioural observations including infant cry, parent-child interaction, social-communication development and language ability during the first and second years of life in high- and low-risk infants. The third aim was to examine, which of these behavioural characteristics of high-risk infants, if any, were predictive of later ASD outcomes at age two. These empirical data are reported on in Chapters 4 and 5. The results of the thesis in its entirety are summarized in Chapter 6 and the theoretical and clinical implications of the findings are also discussed. Appendix I of the thesis reports on empirical evidence to develop a proof of principle study of a “bottom-up” research design.

Table 1. Summary of each studies’ methodology and sample

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<td>Chapter 2</td>
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<td>This study compares HR and LR fetal two-dimensional ultrasound measurements of head and body growth.</td>
<td>17-21 weeks gestation: HR n = 23; LR n = 36</td>
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<td>22-25 weeks gestation: HR n = 17; LR n = 32</td>
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<td>27-32 weeks gestation: HR n = 18; LR n = 35</td>
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<td>Chapter 3</td>
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<td>Comparison of fetal brain growth between HR and LR fetuses at up to two time points during the second trimester of pregnancy.</td>
<td>17-21 weeks gestation: HR n = 15; LR n = 22</td>
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<td>Participants who supplied more than one ultrasound:</td>
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CHAPTER 4

HR n = 13; LR n = 22

Chapter 4

Behavioural observations and parent-report questionnaires were obtained for HR and LR infants at ages 6- and 12-months old. Analyses first compared these measures between HR and LR infants, then turned towards examining where measures collected at 6- and 12-months were predictive of ASD classification at age two.

6-month follow-up:
HR n = 28; LR n = 38
12-month follow-up:
HR n = 28; LR n = 40
Two-year follow-up:
HR n = 31; LR n = 42

Chapter 5

Parents captured multiple recordings of their 12-month-old infant crying over the course of 1-2 weeks. Acoustic properties of the cry episodes (e.g. fundamental frequency and amplitude) were then compared between HR and LR infants. Data were then visually inspected to identify whether infants who met ASD classification at age two displayed a unique acoustic profile at 12-months old.

12-month follow-up:
HR n = 22; LR n = 27
Met ASD classification (ADOS-G) at age two:
HR n = 5; LR n = 1
Did not meet ASD classification (ADOS-G) at age two:
HR n = 16; LR n = 24

Compliance with Ethical Standards

This research was funded by a Project Grant (APP1003424) and Senior Research Fellowship to Professor Whitehouse (supervisor and co-author; APP1077966) from the National Health and Medical Research Council (NHMRC). The PhD candidate would like to acknowledge the participating families who generously donated their time to this study. Professor Hickey (co-author) is funded by an NHMRC Practitioner Fellowship (1058935). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of manuscripts.
Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.
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Foreword to Chapters Two and Three

The review presented in Chapter 1 highlighted that the prenatal period of development has been largely unexplored in relation to risk markers for ASD. The first aim of this thesis was to shed some light on early prenatal brain development to provide a further examination of the brain overgrowth hypothesis of ASD. The following two chapters were designed to address this gap in the literature. Specifically, Chapters 2 and 3 present data collected at multiple time points during pregnancy using two and three-dimensional methodologies, respectively. Measurements of prenatal head and body growth were then compared between high- and low-risk fetuses to identify potential differences in in utero growth trajectory. Chapter 3 also includes an examination of the association between these prenatal measures and ASD symptomatology in postnatal life.
Numerous studies have observed that a proportion of infants later diagnosed with ASD experience accelerated head growth during the first years of life. An emerging methodology for examining the developmental trajectory prior to a diagnosis of ASD is to investigate siblings of affected individuals. The current study is the first prospective investigation of fetal growth in siblings of children with ASD. Two groups of pregnant women were recruited as part of the PRegnancy Investigation of Siblings and Mothers (PRISM) of children with autism cohort in Perth, Western Australia. The ‘high risk’ (HR) group (n = 23) comprised pregnant women who have an existing child with a diagnosis of ASD and the ‘low risk’ (LR) group (n = 36) comprised pregnant mothers who have an existing child who has developed typically. Prenatal ultrasounds were procured at multiple time-points throughout the second- and third-trimesters, enabling an examination of growth trajectories. Growth measurements were then compared for the HR and LR fetuses. Mixed linear regression models identified no significant differences between the high- and low-risk fetuses in the rate of prenatal head and body growth throughout the second and third trimester (all p-values >.05). Similarly, there were no significant differences observed when comparing HR and LR groups on a ratio of head circumference relative to body size (β= -.019, p=.75). Future studies may consider looking beyond the macro architecture of the prenatal brain and examine the growth of brain sub-regions that have been implicated in the presentation of ASD symptoms.

Numerous studies have identified accelerated head growth during the first year of life among infants subsequently diagnosed with ASD (Redcay & Courchesne, 2005; Courchesne, Campbell & Solso, 2011), though recent investigations have reported inconsistency in this pattern of findings (Zwaigenbaum et al., 2014). Head circumference (HC) is known to correlate highly with brain volume in early childhood (Bartholomeusz, Courchesne, & Karns, 2002) and accelerated HC growth is thought to
reflect abnormal brain volume in children with ASD. Several studies have also reported that macrocephaly is observed among newborns later diagnosed with ASD at twice the prevalence of the typical population (Gillberg & De Souza, 2002; Hobbs et al., 2007), suggesting that atypical neurodevelopment may commence prenatally in some cases of ASD. However, again, these findings have not always been replicated (Redcay & Courchesne, 2005).

Two studies have examined prenatal head circumference growth in children with ASD. Hobbs et al. (2007) retrospectively obtained second trimester fetal ultrasound data of 45 individuals with ASD and 222 typically developing control children. Children with ASD did not differ significantly from control children on measurements of head circumference, biparietal diameter, abdominal circumference, and femur length recorded at a mean of 19 weeks gestation. However, an index of head shape, the mean biparietal diameter relative to head circumference, was found to be significantly increased in the ASD group, indicating a subtle disturbance in the uniformity of fetal brain growth. A second study examined second trimester ultrasound data obtained in a prospective study of a large general population pregnancy cohort (Whitehouse et al., 2011). Fourteen children with ASD were each matched with four typically developing control children (N =56) and head circumference at 18 weeks gestation and birth were compared between groups. There were no significant differences between groups in head circumference size at either time point, though it was noted that 4 of the 11 (36.4%) children with ASD met a predefined threshold for a large head compared to body size (compared to 0% of the control group).

An emerging methodology for examining the developmental trajectory prior to a diagnosis of ASD is to investigate siblings of affected individuals. There are two benefits of this approach. First, siblings of ASD probands are at increased risk for disorder themselves; it has been estimated that around 18.7% of siblings meet diagnostic criteria for ASD (Ozonoff et al., 2011), and a further 20% show more subtle communication/social impairments or stereotypic behaviours (Constantino et al., 2010; Risch et al., 2014; Zwaigenbaum et al., 2009). Second, because biological siblings share half of their segregating genes on average, siblings of children with ASD are likely to share a subset of the susceptibility genes that lead the proband to express the condition. Therefore, phenotypic features potentially associated with ASD, such as brain overgrowth, are also more likely to be present in relatives, albeit potentially reduced in magnitude.
The current study is the first prospective investigation of fetal growth in siblings of children with ASD. Prenatal ultrasounds were procured at multiple time-points throughout the second- and third-trimesters facilitating an examination of growth trajectories. Growth measurements of the ASD sibling fetuses (‘high risk’ (HR) fetuses) were then compared with measurements for a control group, comprising sibling fetuses of typically developing children (‘low risk’ (LR) fetuses). Given the few studies to date on prenatal measures of growth, no hypotheses were proposed.

Method

Participants

Participants were part of the PRegnancy Investigation of Siblings and Mothers (PRISM) of children with ASD cohort in Perth, Western Australia, which is a longitudinal study of pregnant women recruited during pregnancy. Two groups of pregnant women were recruited. The HR group comprised women with an existing biological child who had received a clinical diagnosis of either Autistic Disorder or Pervasive Developmental Disorder-Not Otherwise Specified according to DSM-IV criteria (APA, 1994) (the ‘proband’). In Western Australia, a diagnosis of ASD mandates consensus by a team comprising a Pediatrician, Psychologist and Speech-Language Pathologist. The LR group comprised women with an existing biological child who was at least three years of age and had received no diagnosis of a developmental disorder. Women were recruited to the study via advertisements in local newspapers or referrals from their obstetricians or gynaecologists. While women were preferentially recruited prior to 18 weeks pregnancy, women were enrolled in the study if they agreed to participate at any time throughout their pregnancy. The final sample size for analysis comprised 23 ‘high-risk’ pregnancies and 36 ‘low-risk’ pregnancies.

Recruitment and assessment

Families were invited to the Telethon Kids Institute (University of Western Australia) for a face-to-face behavioural assessment. Mothers were asked to complete a comprehensive case-history questionnaire about their pregnancy, family and medical history, and the proband child’s development.
Probands in the high-risk group were administered the Autism Diagnostic Observation Schedule-Generic as part of the study protocol (ADOS-G; Lord et al., 2000). All but five of the probands met criteria for ASD on the ADOS-G. Given the rigorous nature of clinical diagnostics in Western Australia, and that several years may have passed since the original clinical diagnosis during which therapeutic benefits may have mitigated ASD behaviours, we decided to include all participants in further analyses. Motor skill and language development were also assessed in proband children using the Mullen Scales of Early Learning (MSEL; Mullen, 1995).

**Fetal ultrasound studies**

Participation in the PRISM study involved fetal ultrasound imaging studies at three time-points throughout pregnancy: 17-21 weeks, 22-25 weeks and 27-32 weeks gestation. Depending on the week of pregnancy at which a woman was enrolled, mothers underwent one, two or three of these scans conducted by experienced ultrasonographers. These time points were selected based on our pilot work to maximize the time between scans in the second trimester, while minimizing measurement artefacts that increase as pregnancy progresses. Gestational age was calculated from the last menstrual period (LMP) and later confirmed via ultrasound using measurements of crown-rump length taken in the first trimester in reference to the Robinson charts (Robinson, 1973). Where there was a significant discrepancy between GA estimates, the estimate based on crown-rump length was preferred given that LMP estimates can be influenced by factors such as menstrual cycle length and the use of the contraceptive pill. We do not anticipate that the use of crown-rump length during the first trimester to calculate GA will have a significant influence on the current study, given that our key outcome variables are measurements of different growth parameters (head growth) and at a later time point (second and third trimesters).

All ultrasound examinations were completed by one of two experienced sonographers using one of two Voluson E8 machines and C1-5 and RAB 4-8 3D/4D transducers. Fetal brain size was estimated by measuring the maximal head circumference (HC), as well as the maximum occipitofrontal (OFD) and biparietal (BPD) distances using standard procedures (Whitehouse et al., 2011). Overall fetal size was indexed by femur length (FL). All measurements were to the nearest millimetre.
Previous studies have examined the reproducibility of fetal biometry and found evidence that inter-observer reliability is very high between experienced sonographers (Perni et al., 2004), particularly during the first two prenatal trimesters (Sarris et al., 2012). In the current study, there were no significant differences between groups in the proportion of scans completed by each sonographer. Fetal ultrasound under standard conditions is considered a safe procedure that can be carried out multiple times throughout pregnancy (Newnham et al., 1993). Several large studies have found no association between the frequency of prenatal ultrasound during pregnancy and offspring ASD (Grether et al., 2010; Stoch et al., 2012).

**Statistical analysis**

Analyses first concentrated on comparing the characteristics of the probands and family demographics of the HR and LR groups using chi-square (categorical variables) and independent samples t-tests (continuous variables). Our analyses then turned to the HC, OFD, BPD and FL ultrasound measurements. To examine HC relative to body size, which has been a key feature of previous studies (e.g. Whitehouse et al., 2011), three ratio estimates were calculated by dividing HC, BPD and OFD by FL. A higher score indicated greater HC, BPD or OFD relative to body size. HC uniformity was also calculated by dividing HC by BPD, and HC by OFD.

To examine if there were differences between the HR and LR groups on the ultrasound measures we analysed the data using two linear mixed effect models for each measurement outcome. The first model was designed to examine the difference between the HR and LR groups in measurements of HC, OFD, BPD and FL. The second model was designed to examine whether there were any differences between the two groups when looking at growth rate throughout pregnancy.

**Results**

Characteristics of the probands are presented in Table 2 and family demographics are reported in Table 3. There were no significant differences between groups in the number of ultrasounds received nor the proportion of males and females.
Table 2. Characteristics of probands in the two groups

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group (N = 23)</th>
<th>‘Low risk’ group (N = 36)</th>
<th>( \chi^2 )</th>
<th>( p )</th>
<th>( \phi )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18 (78)</td>
<td>17 (47)</td>
<td>5.60</td>
<td>&lt;.02</td>
<td>.31</td>
</tr>
<tr>
<td>Female</td>
<td>5 (22)</td>
<td>19 (53)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ADOS classification</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism</td>
<td>15 (65)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism spectrum</td>
<td>3 (13)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4 (17)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did not complete</td>
<td>1 (4)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ADOS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at assessment</td>
<td>6.73 (5.70)</td>
<td>2.51 (1.21)</td>
<td>4.31</td>
<td>&lt;.001</td>
<td>1.02</td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELC standard score</td>
<td>75.25 (25.93)</td>
<td>106.89 (17.27)</td>
<td>-</td>
<td>&lt;.001</td>
<td>1.43</td>
</tr>
<tr>
<td>WISC</td>
<td>n = 7</td>
<td>n = 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full scale IQ</td>
<td>85.71 (36.71)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Characteristics of the family, the pregnancy and the fetus.

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group (N = 23)</th>
<th>‘Low risk’ group (N = 36)</th>
<th>$\chi^2$</th>
<th>$p$</th>
<th>$\varphi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal education</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did not complete secondary</td>
<td>1 (5)</td>
<td>0 (0)*</td>
<td>4.23</td>
<td>.12</td>
<td>.28</td>
</tr>
<tr>
<td>school</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Completed secondary school</td>
<td>8 (38)</td>
<td>7 (20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Completed university degree</td>
<td>12 (57)</td>
<td>28 (80)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paternal education</td>
<td></td>
<td></td>
<td>1.91</td>
<td>.39</td>
<td>.18</td>
</tr>
<tr>
<td>Did not complete secondary</td>
<td>1 (5)</td>
<td>0 (0)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>school</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Completed secondary school</td>
<td>11 (52)</td>
<td>17 (49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Completed university degree</td>
<td>9 (43)</td>
<td>18 (51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal smoking during</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal alcohol intake at</td>
<td>1 (4)</td>
<td>0 (0)</td>
<td>1.55</td>
<td>.21</td>
<td>-.16</td>
</tr>
<tr>
<td>least weekly during pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age at conception</td>
<td></td>
<td></td>
<td>4.32</td>
<td>.04</td>
<td>-.27</td>
</tr>
<tr>
<td>20-35 years</td>
<td>9 (39)</td>
<td>24 (67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>14 (61)</td>
<td>12 (33)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paternal age at conception</td>
<td></td>
<td></td>
<td>1.88</td>
<td>.17</td>
<td>-.18</td>
</tr>
<tr>
<td>20-35 years</td>
<td>7 (30)</td>
<td>17 (49)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>16 (70)</td>
<td>18 (51)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N prenatal ultrasounds received</td>
<td></td>
<td></td>
<td>.62</td>
<td>.73</td>
<td>.10</td>
</tr>
<tr>
<td>1</td>
<td>5 (22)</td>
<td>5 (14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5 (22)</td>
<td>9 (25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13 (56)</td>
<td>22 (61)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex of fetus</td>
<td></td>
<td></td>
<td>.00</td>
<td>.96</td>
<td>.05</td>
</tr>
<tr>
<td>Male</td>
<td>11 (48)</td>
<td>17 (47)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12 (52)</td>
<td>19 (53)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Missing data for one family
The descriptive statistics of the fetal measurements at the three time points are presented in Table 4. Gestational age at time of ultrasound did not differ significantly between the HR and LR groups. Mixed model regression analyses were performed to examine growth parameters between the HR and LR groups. Outcomes of the tests for group differences, adjusting for gestational age, are displayed in
Table 5. The positive beta values indicate that LR measurements were slightly larger than HR measurements though these differences did not achieve statistical significance.

A second set of mixed model regression analyses was performed examining the interaction of high- versus low-risk group with gestational age (HC, $\beta = -.15$, $p = .56$; BPD, $\beta = .03$, $p = .74$; OFD, $\beta = -.14$, $p = .28$; FL, $\beta = -.02$, $p = .78$). The negative beta value for HC, OFD and FL indicates slightly faster growth rate for these measurements in the HR group relative to the LR group, though again this did not reach statistical significance.

Further analyses revealed no significant differences in the head size/femur length ratios either in the simple model or for the growth rate model (all $p$-values $>.05$, See Figure 1). There were also no significant high- versus low-risk group differences in HC uniformity for BPD ($\beta = .02$, $p = .40$) or for OFD ($\beta = .02$, $p = .32$). Mixed model regression analyses were also performed excluding proband families who did not meet ADOS-G criteria for ASD (all proband participants have been diagnosed according to rigorous standards see Method section). When excluding those five families, there were no significant differences in growth measurements between high- and low-risk fetuses.

Table 4. *Descriptive statistics showing the M (SD) gestation age in weeks, and fetal*
measurements (in mm) obtained at each time point.

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group</th>
<th>‘Low risk’ group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 23)</td>
<td>(N = 36)</td>
</tr>
<tr>
<td><strong>M (SD)</strong></td>
<td><strong>M (SD)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>17-21 weeks gestation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Week</td>
<td>18.24 (.77)</td>
<td>18.64 (.86)</td>
</tr>
<tr>
<td>Head circumference</td>
<td>156.90 (11.07)</td>
<td>162.68 (11.27)</td>
</tr>
<tr>
<td>Occipitofrontal diameter</td>
<td>55.35 (4.00)</td>
<td>56.96 (3.93)</td>
</tr>
<tr>
<td>Biparietal diameter</td>
<td>42.90 (2.83)</td>
<td>44.20 (3.20)</td>
</tr>
<tr>
<td>Femur length</td>
<td>27.81 (3.17)</td>
<td>29.60 (2.26)</td>
</tr>
<tr>
<td><strong>22-25 weeks gestation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>17</td>
<td>32</td>
</tr>
<tr>
<td>Week</td>
<td>23.94 (.75)</td>
<td>23.97 (.47)</td>
</tr>
<tr>
<td>Head circumference</td>
<td>217.24 (10.84)</td>
<td>221.13 (9.78)</td>
</tr>
<tr>
<td>Occipitofrontal diameter</td>
<td>76.18 (4.79)</td>
<td>78.47 (4.02)</td>
</tr>
<tr>
<td>Biparietal diameter</td>
<td>59.71 (3.50)</td>
<td>60.03 (3.63)</td>
</tr>
<tr>
<td>Femur length</td>
<td>43.29 (2.93)</td>
<td>43.94 (3.31)</td>
</tr>
<tr>
<td><strong>27-32 weeks gestation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>18</td>
<td>35</td>
</tr>
<tr>
<td>Week</td>
<td>28.06 (1.21)</td>
<td>28.20 (.72)</td>
</tr>
<tr>
<td>Head circumference</td>
<td>259.72 (19.43)</td>
<td>264.86 (12.39)</td>
</tr>
<tr>
<td>Occipitofrontal diameter</td>
<td>93.22 (5.62)</td>
<td>92.76 (4.74)</td>
</tr>
<tr>
<td>Biparietal diameter</td>
<td>71.33 (4.73)</td>
<td>72.77 (3.43)</td>
</tr>
<tr>
<td>Femur length</td>
<td>53.39 (2.77)</td>
<td>54.43 (3.28)</td>
</tr>
</tbody>
</table>
Table 5. Tests of the Effect of High- versus Low-risk Group using Linear Mixed Model Regression Analyses of HC, OFD, BPD and FL Adjusted for Gestational Age.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>(\beta^a)</th>
<th>(p)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mm)</td>
<td>Lower Bound</td>
<td>Upper Bound</td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>3.76</td>
<td>.17</td>
<td>-1.69</td>
</tr>
<tr>
<td>OFD</td>
<td>.62</td>
<td>.48</td>
<td>-1.14</td>
</tr>
<tr>
<td>BPD</td>
<td>.54</td>
<td>.42</td>
<td>-.78</td>
</tr>
<tr>
<td>FL</td>
<td>.73</td>
<td>.18</td>
<td>-.33</td>
</tr>
<tr>
<td>HC/FL</td>
<td>-.03</td>
<td>.57</td>
<td>-.15</td>
</tr>
<tr>
<td>OFD/FL</td>
<td>-.02</td>
<td>.31</td>
<td>-.07</td>
</tr>
<tr>
<td>BPD/FL</td>
<td>-.02</td>
<td>.31</td>
<td>-.05</td>
</tr>
<tr>
<td>HC/OFD</td>
<td>.02</td>
<td>.32</td>
<td>-.02</td>
</tr>
<tr>
<td>HC/BPD</td>
<td>.02</td>
<td>.40</td>
<td>-.03</td>
</tr>
<tr>
<td>Model 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>-.15</td>
<td>.56</td>
<td>-.66</td>
</tr>
<tr>
<td>OFD</td>
<td>-.14</td>
<td>.28</td>
<td>-.40</td>
</tr>
<tr>
<td>BPD</td>
<td>.03</td>
<td>.74</td>
<td>-.13</td>
</tr>
<tr>
<td>FL</td>
<td>-.02</td>
<td>.78</td>
<td>-.18</td>
</tr>
<tr>
<td>HC/FL</td>
<td>.02</td>
<td>.09</td>
<td>-.00</td>
</tr>
<tr>
<td>OFD/FL</td>
<td>.00</td>
<td>.29</td>
<td>-.00</td>
</tr>
<tr>
<td>BPD/FL</td>
<td>.01</td>
<td>.09</td>
<td>-.00</td>
</tr>
<tr>
<td>HC/OFD</td>
<td>.00</td>
<td>.20</td>
<td>-.00</td>
</tr>
<tr>
<td>HC/BPD</td>
<td>-.00</td>
<td>.55</td>
<td>-.01</td>
</tr>
</tbody>
</table>

\(a\) Beta is the adjusted mean difference in measurement between groups. A positive beta value indicates low-risk measurements are larger compared to high-risk measurements.
Discussion

The current study observed no significant differences between the HR and LR fetuses on ultrasound measurements of HC, BPD, OFD or FL, nor were there any significant differences in the rate of prenatal head and body growth throughout the second and third trimester. Analyses of HC uniformity and ratios of head growth relative to body size also revealed no differences between the HR and LR fetuses.

The null findings are broadly consistent with retrospective studies of children later diagnosed with ASD, which found that children with ASD did not differ significantly from control children on prenatal measurements of HC, BPD, AC, and FL (Hobbs et al., 2007; Whitehouse et al., 2011). Previous studies had identified some subtle disturbances in the uniformity of fetal brain growth in the ASD group. For example, Whitehouse et al. (2011) noted that several children with ASD had a relatively large head compared to body size. Whilst the current study observed no such difference
in the high-risk fetuses, any growth abnormalities may be more subtle in a sibling cohort, and more readily identified in the fetuses that are later diagnosed with ASD.

The key strengths of this study lie in the closely matched participant samples that are difficult to recruit and follow longitudinally. Ultrasounds were collected at multiple time-points, which enabled both cross-sectional and longitudinal analyses of fetal growth. Several participants in the current study were unable to attend all three of their ultrasound appointments or were recruited late in their second or third trimester, which is suboptimal for Doppler anthropometric measurement. While these issues reduced the number of scans included in the trajectory analyses, multiple scans (using the same machine and sonographer) were available from 78% and 86% of the HR and LR samples, respectively.

This study was the first prospective investigation of fetal growth in siblings of children with ASD, and opens up several avenues for future research. The study had a relatively small sample size, and while no comparison achieved statistical significance, the mixed model beta values indicated consistent statistical trends for the HR group to have slightly smaller growth measurements but faster growth rate relative to the LR group. The further investigation of more HR fetuses, and potential meta-analysis with the current data, will help to elucidate the clinical significance, if any, of these observations. The continual follow-up of these fetuses to a postnatal age at which ASD diagnostic behaviours can be appraised will also help to determine whether differences are observed when samples are restricted to fetuses who receive a diagnosis of ASD. Again, larger sample sizes, potentially collected across multiple research teams, are critical to achieving this research aim. Finally, future research may consider going beyond routine ultrasound biometry and examine the growth of sub-regions in the brain that are thought to be implicated in the presentation of ASD symptoms, such as the corpus callosum (e.g. Booth et al., 2011; Lefebvre, Beggiato, Bourgeron, & Toro, 2014) or cerebellum (e.g. Fatemi et al., 2012). Fetal functional magnetic resonance imaging as well as 3D ultrasonography technology (Studholme, 2011; Timor-Tritsch, Monteagudo, Pilu, & Malinger, 2012) enable more fine-grained analysis of prenatal cortical growth. Studies that capitalize on these imaging advancements may build on the null findings presented here and thereby identifying more subtle differences in neural growth of fetuses at high risk of ASD.
References


CHAPTER 3  A preliminary study of cerebral growth in fetuses at high-risk of Autism Spectrum Disorder

Previous research has indicated that an atypical pattern of brain morphology growth may precede a diagnosis of autism spectrum disorder (ASD). While there is preliminary evidence that atypical neurodevelopment in ASD may commence prenatally, no study has directly examined brain volume growth during fetal life. The current study prospectively measured cerebral growth during the second prenatal trimester in siblings of children with ASD. The high-risk group (n = 19) comprised pregnant women who have an existing child with ASD. The low-risk group (n = 31) comprised pregnant women with no family history of ASD. Fetal brain growth was examined using three-dimensional ultrasound collected by experienced ultrasonographers blind to participant group. Ultrasounds were procured at up to two time-points throughout the second-trimester facilitating an examination of growth trajectories. Cerebral volume measurements were compared between the high- and low-risk fetuses using regression modelling. We also examined data points of the individuals who either had a clinical diagnoses of ASD (n = 3) or displayed behavioural risk for ASD (n = 1) at the 2 year postnatal follow-up. High-risk fetuses demonstrated an atypical pattern of cerebral growth across the second prenatal trimester. Whereas the low-risk fetuses exhibited a linear increase in brain volume across the second trimester, the high-risk fetuses showed a positively accelerated quadratic function over this period, characterised by a slow rate of growth between 17 and 21 weeks’ gestation and a more rapid rate of growth from 22 weeks onwards (p = .027 for the quadratic trend). However, the data points for the four individuals who displayed the ASD phenotype at age two years did not appear to drive this between-groups difference. This study is the first to examine cerebral volume in fetuses at high-risk of ASD. This small study observed a preliminary finding of an atypical growth trajectory between high-risk and low-risk fetuses, though we note that the individuals who were diagnosed with clinical ASD did not appear to contribute to this atypical trajectory. Future studies are required to further elucidate these observations of atypical brain growth during the second prenatal trimester.
A widely reported observation in the study of autism spectrum disorder (ASD) is the presence of accelerated head circumference growth during infancy and toddlerhood, which is followed by deceleration in growth throughout the adolescent years and into early adulthood (Courchesne, Carper, & Akshoomoff, 2003; Gillberg & Souza, 2002; Redcay & Courchesne, 2005). While these findings have been interpreted as differences in the rate of cerebral growth, studies that have used more direct measurements of the cortex have cast doubt on this phenomenon (Shen et al., 2013; Zwaigenbaum et al., 2014). Using Magnetic Resonance Imaging (MRI), Shen et al. (2013) observed no differences in cerebral volume between 6-month-old infants who were developmentally delayed, diagnosed with ASD or typically developing at 36 months, indicating that this pattern of cerebral growth may not be as pervasive in the ASD population as first thought. Raznahan et al. (2013) investigated these inconsistencies across studies by performing a systematic review examining the early brain overgrowth in ASD. They concluded that several of the reference norms, which have been previously used to determine head circumference overgrowth in ASD, may be biased toward detecting HC overgrowth in samples of typically developing children (Raznahan et al., 2013).

Despite the inconsistent findings, there remains considerable interest in early brain volume growth in children diagnosed with ASD (Courchesne, Campbell, & Solso, 2011). Several key findings have led researchers to hypothesize that the process of brain overgrowth in children with ASD may begin prenatally. For example, observations of macrocephaly have been reported among newborns later diagnosed with ASD at twice the prevalence of typically developing children (Gillberg & Souza, 2002). Furthermore, two retrospective studies examining fetal biometry from prenatal ultrasound in children with ASD have provided preliminary evidence for subtle differences in the uniformity of fetal brain size in children with ASD (Hobbs et al., 2007; Whitehouse et al., 2011). More recently, Stoner et al. (2014) obtained post-mortem neocortical tissue samples from children aged 2-15 years and used RNA in situ hybridization to analyse cortical microstructure. Cortical disorganization of neurons and atypical laminar cytoarchitecture was observed in the prefrontal and temporal cortical tissue from 10 out of 11 children diagnosed with ASD, compared with only 1 of the 11 typically developing children. Stoner et al. (2014) suggested that this observation may point to dysregulation in fetal neural development during pregnancy.

Prospective studies of siblings of ASD probands provide another methodology through which the fetal neurodevelopment in ASD can be investigated. Siblings of ASD
probands have an estimated 18.7% increased risk for disorder themselves (Ozonoff et al., 2011), and since biological siblings share approximately 50% of their segregating genes, it is more likely that siblings will share a proportion of the susceptibility genes that are implicated in the probands’ ASD (Ozonoff et al., 2011). To date, only one study, conducted by our team (Unwin et al., 2016), has examined prenatal growth in an infant sibling cohort. Pregnant women who had an existing child with ASD (the ‘high risk’ (HR) group) or an existing child with no family history of ASD (the ‘low risk’ (LR) group) were recruited and fetal biometrics (e.g., head circumference, biparietal diameter and occipital-frontal diameter) were obtained at three time-points throughout pregnancy using routine prenatal ultrasound. There were no statistically significant differences in head size measurements and growth trajectories across the second prenatal trimester for HR sibling fetuses relative to the LR sibling fetuses. Whilst this finding contrasts with other evidence of atypical prenatal neurodevelopment in ASD (Gillberg & Souza, 2002; Hobbs et al., 2007; Whitehouse et al., 2011), it is possible that any differences in cerebral growth within a sibling cohort may be too subtle to be measured using routine fetal biometry (Unwin et al., 2016).

Three-dimensional ultrasound has emerged over the past decade as a powerful obstetric technique to analyse fetal growth, including cortical morphology. Volumetric measurements using antenatal three-dimensional ultrasound have excellent reproducibility (Chang, Yu, Chang, Ko, & Chen, 2003; Miguelote, Vides, Santos, Matias, & Sousa, 2012), are highly correlated with routine fetal biometry (ICC >.9; Roelfsema, Hop, Boito, & Wladimiroff, 2004) and correlations with structural magnetic resonance imaging (MRI), which is the gold standard for fetal brain measurement, are excellent (i.e. $r > .90$) (Haratz et al., 2011). This study presents prenatal brain growth data collected via three-dimensional ultrasound. Prenatal ultrasound was performed at up to two time-points during the second-trimester, facilitating observations of cerebral growth trajectories. Trajectories of brain volume growth were then compared between HR and LR fetuses. Follow-up data of HR and LR fetuses up to the postnatal age of two years enabled us to identify those individuals with a clinical diagnosis of ASD, and also those who were showing subthreshold behavioural risk factors. Given the limited research on prenatal measures of growth in at-risk siblings, no hypotheses were proposed.
Methods

Participants

Participants were part of the ‘PRegnancy Investigation of Siblings and Mothers (PRISM) of children with ASD’ at the Telethon Kids Institute (Perth, Western Australia). PRISM is a prospective longitudinal study of two groups of women; 1) the ‘high risk’ (HR) group of pregnant women with an existing, biological child who has been diagnosed with ASD (the ‘proband’), and 2) the ‘low risk’ (LR) group of pregnant women with no family history of ASD and an existing child who was at least two years of age. A further inclusion criteria for the LR group was that the pregnant woman and the father of the LR fetus had no first-degree family member diagnosed with a neurodevelopmental disorder. Participants were recruited to the study via referrals from their obstetricians or gynaecologists or from local advertisements. While women were preferentially recruited prior to 18 weeks gestation, enrolment was open at any time throughout pregnancy. Three-dimensional ultrasound measurements for analysis were available for 19 HR singleton pregnancies and 31 LR singleton pregnancies. Informed consent was obtained from all individual participants included in the study.

Recruitment and assessment

Families were invited to the Telethon Kids Institute and asked to complete a detailed questionnaire about their medical, pregnancy and family history, as well as provide details on the early behavioural development of their proband child. Clinical ASD diagnoses were confirmed following the administration of the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000) for all but four of the HR probands. Given the rigorous nature of clinical diagnostics in Western Australia (Glasson et al., 2008), and that therapeutic benefits may have mitigated ASD behaviours in the years since the original diagnosis, all participants were included in further analyses. In all four cases, the study investigators sourced the original diagnostic report and ASD diagnoses were confirmed. The proband child’s language and motor skill development were also assessed using the Mullen Scales of Early Learning (MSEL) (Mullen, 1995).

At postnatal follow-up of HR and LR fetuses at age two, three of the HR infants had received a clinical diagnosis of ASD, provided by a multidisciplinary team
comprising a speech pathologist, paediatrician, and clinical psychologist. ASD diagnosed were also confirmed via administration of the ADOS-G. Given that there is still variability in the behavioural phenotype of ASD at age two (Charman et al., 2005; Cox et al., 1999), we also administered the Communication and Symbolic Behavior Scales – Developmental Profile (CSBS DP; Wetherby & Prizant, 2002) to indicate behavioral risk for a future diagnosis of ASD. The CSBS DP is a standardized 24-item parent-report checklist of social, symbolic and communication abilities of children aged between 6-24 months old. This tool has been validated as a first-level screener of ASD for children who score in the bottom 10th percentile on the Checklist (Wetherby et al., 2004). In the current study, individuals without a diagnosis who had a score in the bottom 10th percentile, relative to norms of age-matched peers, were considered to be at ‘behavioural risk’ of a later diagnosis of ASD.

Fetal ultrasound

Fetal ultrasound imaging was performed at two time-points during the second trimester of pregnancy: 17-21 weeks and 22-25 weeks. Mothers underwent one or two of these scans, depending on the week of pregnancy at which they enrolled in the PRISM study. In reference to the Robinson charts, ultrasound measurements of the first trimester crown-rump length were used to determine the gestational age of the fetus (GA) (Robinson, 1973). This method of GA estimate was preferred given that last-menstrual-period estimates can vary according to factors such as use of hormonal contraception and length of menstrual cycle (Lynch & Zhang, 2007).

All ultrasound examinations were completed by one of two experienced ultrasonographers (W.L. and M.B.), who were blind to participant group. The ultrasonographers used one of two Voluson E8 ultrasound machine systems and C1-5 and RAB 4-8 3D/4D transducers (also see Unwin et al., 2016). Measurements were obtained from each fetus when there was little to no fetal movement, and the images were stored to facilitate inter-rater reliability calculations. The total cerebral volume was obtained from the biparietal diameter plane, which included the falx cerebri anteriorly and posteriorly, and the cavum septi pellucidi and thalamic nuclei in the midline. Fetal cerebral volume was calculated using the VOCAL method (GE Healthcare) (Bordes, Bory, Benchaib, Rudigoz, & Salle, 2002; Raine-Fenning et al., 2002). The VOCAL 3DUS method involves the use of specific 4D software and rotates the structure around its own axis using a pre-determined angle selected by the sonographer. The outer
contours of consecutive planes are then manually traced on the screen to obtain area measurements (Barreto et al., 2010). Once this process is complete, the software automatically calculates the volume; reconstructing the selected object (e.g. cerebrum) in a 3D format. Barreto et al. (2010) recently examined the reliability and validity of this method by asking two ultrasonographers to obtain volumes of gel-filled 3D objects of different shapes and sizes. They reported that the VOCAL method had good interobserver and intraobserver reproducibility, irrespective of the object that was measured. They reported a slight overestimation of the object volume by 2.8%, but this overestimation was stable across measurements (Barreto et al., 2010). They argued that because 3D ultrasound allows the correct evaluation of objects with irregularities, it produces more reliable volume estimates than 2D ultrasound and is sufficiently accurate to be used in clinical practice.

In the current study, six images were used to trace the outline of the cerebrum which was acquired when the rotation angle was specified at 30° and the axial view was selected as the reference plane. Frontal region boundaries were defined laterally and anteriorly according to the inner wall of the skull, posteriorly by the Sylvian fissure and inferiorly by the base of the skull. Once the rotational process was completed, the VOCAL software automatically calculated the cerebral volume and generated reconstructed 3D images. Volumes are expressed in cm$^3$. The proportion of scans completed by each ultrasonographer was comparable for the HR and LR groups ($\chi^2(1) = .32, p = .58$). Importantly, Newnham et al. (1993) reported evidence that routine fetal ultrasound can safely be administered multiple times during pregnancy and several large studies have also reported no association between prenatal ultrasound and ASD risk in offspring (Grether et al., 2010; Stoch et al., 2012).

**Statistical Analyses**

Chi-square analyses (categorical variables) and independent-samples t-tests (continuous variables) were used to compare the characteristics of the probands and family demographics of the HR and LR groups. Our analyses then turned to cerebral volume measurements. To examine the reliability of these measurements, 20 scans from each of the prenatal time windows were randomly selected and the cerebral volume was remeasured by the alternative sonographer using VOCAL software in offline mode.
Intraclass Correlation Coefficients (ICC) were calculated to assess the agreement between the two sonographers for measurements of fetal brain volume.

Approximately 70% of mothers in the HR and LR groups received more than one ultrasound (see Table 7), and thus contributed two or more data points to the analyses. To maximise the use of these data whilst also accounting for the non-independent nature of the data, we undertook regression analyses in Mplus 7.2 using the Huber-White sandwich estimator (using the TYPE = COMPLEX command, and maximum likelihood-robust estimation) (Muthén & Muthén, 1998). This estimator corrects the standard errors of the regression parameters when observations are non-independent due to their nesting within higher-level units.

Three regression models were generated and examined separately for the two participant groups. The first model (Model 1) simply examined the influence of fetal sex on cerebral volume growth, and acted as a baseline model from which to compare the two subsequent models. The next two models sequentially examined the influence of longitudinal trends on fetal cerebral volume. Since we did not propose any hypotheses, our choice of regression models was based initially on a visual inspection of scatter plots (Figure 2). We ruled out the possibility of a cubic or higher-order polynomial relationship for conceptual reasons; such relationships, which imply the existence of flexion points, would suggest that there is a point where cerebral volume decreases over time. Thus, our second model (Model 2) examined the additional variance accounted for by a linear growth trend, and our third model (Model 3) examined the additional variance (beyond Model 2) accounted for by a quadratic growth trend. Given that we would only expect positive growth in cerebral volume over time (i.e. we would not expect negative linear or inverted-U quadratic trends), one-tailed tests were conducted with an alpha level set at $p < .05$.

**Results**

Proband characteristics are presented in Table 6 and family demographics and fetus-specific data are reported in Table 7. As expected, based on the higher incidence in ASD of males compared to females (Werling & Geschwind, 2013), 79% of the HR proband group were male, which compared with 50% of siblings in the LR group, $\chi^2(1) = 4.11, p = .04$. Independent-samples t-tests found that HR probands were significantly
older at the time of their assessment ($p < .001$) and scored significantly lower on the MSEL ($p < .001$) compared to LR probands.

Chi-square analyses identified no significant differences between HR and LR groups in maternal or paternal age at birth or maternal smoking or alcohol use during pregnancy (Table 7). Furthermore, there were no statistically significant differences between groups for sex of HR and LR fetuses. The number of ultrasounds received did not differ significantly between the HR and LR groups, nor did fetal gestational age at the time of ultrasound.
Table 6. Characteristics of probands and fetuses in the two groups. ‘High risk’ probands are siblings of fetuses in this group who are diagnosed with ASD, and ‘low risk’ probands are siblings of fetuses in this group who are typically developing.

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group</th>
<th>‘Low risk’ group</th>
<th>χ²</th>
<th>p</th>
<th>φ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 19)</td>
<td>(N = 31)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetus sex</td>
<td></td>
<td></td>
<td>4.11</td>
<td>.04</td>
<td>.30</td>
</tr>
<tr>
<td>Male</td>
<td>15 (79)</td>
<td>15 (48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4 (21)</td>
<td>16 (52)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetus ADOS classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism spectrum</td>
<td>2 (10.5)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TD</td>
<td>15 (79)</td>
<td>31 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetus clinical diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism spectrum</td>
<td>3 (16)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TD /unknown</td>
<td>16 (84)</td>
<td>31 (100)</td>
<td></td>
<td></td>
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<tr>
<td>Proband clinical diagnosis</td>
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<td></td>
</tr>
<tr>
<td>Autism</td>
<td>7 (53)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism spectrum</td>
<td>10 (47)</td>
<td></td>
<td></td>
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<tr>
<td>Proband ADOS classification</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Autism</td>
<td>13 (68)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism spectrum</td>
<td>2 (11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4 (21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proband assessments</td>
<td>M (SD)</td>
<td>M (SD)</td>
<td>t</td>
<td>p</td>
<td>d</td>
</tr>
<tr>
<td>Age at assessment</td>
<td>5.73 (2.17)</td>
<td>2.44 (1.13)</td>
<td>-6.87</td>
<td>&lt; .001</td>
<td>1.90</td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td>n = 13</td>
<td>n = 30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite</td>
<td>73.77 (25.23)</td>
<td>108.23 (17.51)</td>
<td>5.17</td>
<td>&lt; .001</td>
<td>1.57</td>
</tr>
<tr>
<td>Wechsler Intelligence Scale for Children</td>
<td>n = 7</td>
<td>n = 0</td>
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<td></td>
<td></td>
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<tr>
<td>Full scale IQ</td>
<td>85.71 (36.71)</td>
<td></td>
<td></td>
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</table>
Table 7. Characteristics of the family, pregnancy and the fetus

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group (N = 19)</th>
<th>‘Low risk’ group (N = 31)</th>
<th>$\chi^2$</th>
<th>$p$</th>
<th>$\varphi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal smoking during pregnancy</td>
<td>0 (100)</td>
<td>0 (100)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maternal alcohol intake at least</td>
<td>1 (5)</td>
<td>0 (100)</td>
<td>1.61</td>
<td>.21</td>
<td>-.18</td>
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<tr>
<td>weekly during pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age at conception</td>
<td></td>
<td></td>
<td>1.46</td>
<td>.22</td>
<td>-.18</td>
</tr>
<tr>
<td>20-35 years</td>
<td>8 (42)</td>
<td>18 (60)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>11 (58)</td>
<td>12 (40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paternal age at conception</td>
<td></td>
<td></td>
<td>1.14</td>
<td>.29</td>
<td>-.15</td>
</tr>
<tr>
<td>20-35 years</td>
<td>5 (26)</td>
<td>12 (41)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>14 (74)</td>
<td>17 (59)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N prenatal ultrasounds received</td>
<td></td>
<td></td>
<td>.04</td>
<td>.85</td>
<td>.03</td>
</tr>
<tr>
<td>1</td>
<td>6 (32)</td>
<td>9 (29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>13 (68)</td>
<td>22 (71)</td>
<td></td>
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<td></td>
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<tr>
<td>Sex of fetus</td>
<td></td>
<td></td>
<td>.36</td>
<td>.55</td>
<td>.09</td>
</tr>
<tr>
<td>Male</td>
<td>9 (47)</td>
<td>12 (39)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10 (63)</td>
<td>19 (61)</td>
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</tbody>
</table>

*Missing demographic data for one mother and two fathers in the LR group.

For scans collected between 17 and 21 gestational weeks, the two sonographers achieved good overall agreement with an ICC of .86. Similarly, an adequate ICC of .78 was achieved for scans collected between 22 and 25 weeks gestation, where an ICC > 0.7 is commonly used to indicate sufficient reliability (Hripcsak & Heitjan, 2002).

We initially tested whether HR and LR groups shared identical cerebral growth trajectories. To this end, we employed multi-group analyses in Mplus where we specified a quadratic regression model with all parameters constrained to equality across the two groups. The associated statistical test demonstrated that fit for this constrained model was significantly poor ($\chi^2(4, 50) = 10.12, p < .05$). We therefore rejected the null hypothesis that the groups share a common quadratic regression model and respecified separate models for the HR and LR groups.
CHAPTER 3

Inspection of the longitudinal growth data (Figure 2) indicated there was a difference in the growth patterns between the two groups, though it was unclear whether the relationships were of the same form (i.e. linear vs. curvilinear) across the two groups. Initially, we ruled out the possibility of a cubic or higher-order polynomial relationship for conceptual reasons; such relationships, which imply the existence of flexion points, would imply that there is a point where cerebral volume decreases over time. Thus, we initially specified a model including a quadratic term; that is, we modelled cerebral volume as a function of linear gestational age, and the square of the gestational age. We also specified a comparison model which included only a linear term. The results of these analyses are shown in Table 8.

Table 8 The linear regression model accounted for 80.2% of the variance in cerebral volume in the LR group, compared with 90.2% of the variance in the HR group. The addition of a quadratic regression term significantly increased the amount of variance explained in HR cerebral volume by 1.4% (p = .047). By contrast, in the LR group, the quadratic term barely impacted upon the model at all (ΔR2 = .000, p = .993). Quadratic regression models from Table 8 are presented graphically in Figure 2. Collectively, the results suggest the presence of an atypical growth pattern in the HR group, characterized by the right side of a U-shape. Indeed, the quadratic term explained an additional 1.4% of the variance in cerebral volume which can be considered substantial when taken as a proportion of the 10% variance left unexplained by the linear function. By contrast, the LR group showed no signs of any curvilinear relationships between gestational age and cerebral volume.

Upon visual inspection of the data, the quadratic effect observed in the HR fetus group appeared to be driven by measurements at week 20 and 23 (see Figure 2). At age two years, 3 of the 19 HR participants met criteria for ASD (and had received a clinical diagnosis).
Table 9 presents the four data points for these three individuals (also see Figure 2), along with the two data points of the further individual who displayed ‘behavioural risk’ for ASD as measured by the parent-report CBCS measure. Two data points from individuals with a clinical diagnosis of ASD were recorded at 23 weeks, but neither of these data points appeared to be influential in generating the quadratic effect observed.

Table 8. Multi-level linear (Model 1) and quadratic (Model 2) regression models for high- and low-risk groups.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>SE B</th>
<th>p</th>
<th>R²</th>
<th>R² Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linear Model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>High risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant</td>
<td>-</td>
<td>15.07</td>
<td>.</td>
<td>.</td>
<td>.002</td>
</tr>
<tr>
<td></td>
<td>213.81</td>
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<tr>
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<td>.993</td>
<td>.802</td>
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Table 9. Prenatal brain measurements (cm³) of individuals who, at two years of age, had a clinical diagnosis of ASD (n = 3) or displayed ‘behavioural risk’ as measured by the CSBS DP.

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<tr>
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<td></td>
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<td>Clinical ASD 2</td>
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<tr>
<td>Behavioural risk 1</td>
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Figure 2. Multi-level quadratic regression curves for cerebral volume growth across the second trimester. Measurements from HR fetuses are shown in green and measurements from LR fetuses are shown in blue. The black squares represent HR fetuses who received a clinical diagnosis of ASD at two years old.
Discussion

There is preliminary evidence that atypical neurodevelopment in ASD may commence prenatally (Hobbs et al., 2007; Stoner et al., 2014; Whitehouse et al., 2011). Post-mortem studies have identified abnormal cytoarchitecture in the neocortex, which undergoes important phases of growth during fetal life (Stoner et al., 2014). Prospective and retrospective studies of fetal development have focused on routine ultrasound biometry as a proxy measure of brain growth, but these findings have been inconsistent (Hobbs et al., 2007; Ozonoff et al., 2011; Whitehouse et al., 2011). The current study built on this research by using three-dimensional ultrasound to measure the brain volume of HR and LR fetuses of ASD. A previous study of this cohort identified no statistically significant difference between groups for head size and growth measured via two-dimensional ultrasound during the second trimester (Risch et al., 2014).

In contrast, the current study of cerebral volume indicated an atypical pattern of growth for HR fetuses during the second prenatal trimester. In a small sample of HR fetuses we observed a growth pattern that is comparatively slow during gestational weeks 17-21, which is then followed by an accelerated period of growth up until gestational week 25. The quadratic model explained an additional 1.4% of the variance in the cerebral growth trajectory of the HR group, which represents a substantial proportion (14%) of the variance left unexplained by the linear function. Importantly, however, the quadratic effect observed in the HR group appeared to be generated by a small number of data points obtained at 20 and 23 weeks’ gestation. When we examined the two year postnatal outcomes of the participants, we found that none of the data points from the individuals who had either a clinical diagnosis of ASD or were at behavioural risk of ASD, appeared to be influential in generating the quadratic function.

The difference observed between the HR and LR groups is consistent with some postnatal head circumference observations from ASD (Courchesne et al., 2003; Gillberg & Souza, 2002) but is the first to report on prenatal changes. Not all studies in this area have reported atypical brain growth trajectories in infants later diagnosed with ASD. For example, a recent study of infant siblings of children with ASD (n = 442) (Zwaigenbaum et al., 2014), found that patterns of head circumference growth during
the first three years of life did not significantly predict ASD diagnosis at age three. Nevertheless, the data from the current study suggest that early brain volume growth remains an informative area of research and that future studies will benefit from extending investigation to the prenatal period.

Our preliminary data has identified an area for further investigation, where there may be multiple periods of atypical brain growth in ASD spanning both pre- and postnatal life. Intriguingly, while postnatal studies of infant siblings have found that an atypical head growth trajectory is predictive of a diagnosis of ASD but not necessarily ‘high risk’ status (Courchesne & Pierce, 2005; Shen et al., 2013), the current data on prenatal brain volume growth was found to differentiate HR and LR groups, but not those exhibiting the ASD phenotype in early childhood. The current sample size was too small to conduct statistical analyses for the four participants who displayed high levels of ASD behaviours at age two years. However, visual inspection indicated that the measurement of these individuals did not appear to drive the quadratic effect in the HR group, and could not be readily distinguished from the distribution of data points from the LR group. While the sample size of the current study is small, these data suggest that atypical cortical size and growth trajectory during the second trimester is not present in all individuals who are diagnosed with ASD.

Nevertheless, we believe that the significant difference in growth trajectory between the HR and LR groups provides an interesting observation for the field. Given that siblings of ASD probands, irrespective of phenotype, may display biological differences that can inform our understanding of the aetiological pathways to ASD (Ahmed & Vander Wyk, 2013; Belmonte, Gomot, & Baron-Cohen, 2010; Spencer et al., 2012), this finding requires further investigation. The potential mechanisms that could underpin atypical prenatal neurodevelopment in ASD remain unclear. Courchesne & Pierce (2005) proposed a brain growth dysregulation hypothesis, whereby a period of atypical acceleration in growth is then followed by abnormally slow growth in the prefrontal cortex. Several studies have provided preliminary evidence for this proposal. For example, Casanova, Buxhoeveden, Switala, & Roy (2002) identified the presence of smaller than normal minicolumns in larger than normal post-mortem cerebrums of individuals with ASD compared to individuals without ASD. This suggests that there could be an excess of minicolumns in ASD, which would point to mechanisms involved in regulating symmetrical cell division during the second trimester of pregnancy (Casanova et al., 2002). Furthermore, Stoner et al. (2014) reported post-mortem findings
which identified discrete patches of significantly disorganized cortex in individuals with ASD but not typically developing controls. The patches occurred in both the prefrontal and temporal cortex, which are cortical areas that underpin behaviours critical to an ASD diagnosis, including social-emotional communication function. Another hypothesis stems from observations by Baron-Cohen et al. (2015) of elevated steroidogenic activity in amniotic fluid samples taken from pregnancies that result in offspring who develop ASD. There is evidence to suggest that high levels of testosterone exert a direct and deleterious influence on neurogenesis (Estrada, Varshney, & Ehrlich, 2006; Yang et al., 2002). In vitro and in vivo studies have found that exposure to elevated testosterone concentrations can promote neuronal damage through apoptotic pathways, and may indirectly restrict brain growth by disrupting glucose uptake or angiogenesis (Baron-Cohen et al., 2015). The mean gestational age at which amniotic fluid was sampled in the Baron-Cohen et al. (2015) study was 15 weeks gestation, which is approximately the period in which slower brain cerebral growth was observed in the current HR sample. For ethical reasons, amniocentesis can only be performed for maternal or fetal obstetric indications so amniotic fluid samples were not available for analysis in the present study.

The key strength of this study is the longitudinal design, which enabled the prospective measurement of second-trimester cerebral growth in fetuses at HR of ASD and closely matched control participants. The novel use of three-dimensional ultrasound facilitated the accurate measurement of cerebral growth at up to two time-points, and enabled the current investigation to build substantially on previous studies that used two-dimensional measurements as a proxy for cerebral growth (Hobbs et al., 2007; Ozonoff et al., 2011; Whitehouse et al., 2011). Roelfsema et al. (2004) have previously reported good agreement between 2D and 3D ultrasound measurements of brain volume. However, the non-significant result in the 2D ultrasound study of this cohort could be explained in that it only reported on head circumference measurements and did not estimate brain volume. Also, whilst 2D and 3D ultrasound have been shown to be highly correlated, Barreto et al. (2010) discuss how 3D ultrasound allows the correct evaluation of objects with irregularities and thus produces more reliable volume estimates than 2D ultrasound. Volumetric measurements calculated by 3D ultrasound are also known to have excellent correlations with measurements calculated with MRI (Haratz et al., 2011). In the current study, ultrasound data collection was performed by two experienced sonographers, and inter-rater reliability was adequate. A significant
limitation of this study was the small sample size in the HR group, which can be attributed to the challenges with recruiting high-risk ASD cohorts during pregnancy. Prenatal data collection in the current study was limited to the second-trimester, which was a decision based on pilot testing that found Doppler ultrasound had suboptimal validity in cerebral measurements beyond 26 weeks’ gestation. Future studies can build on the data presented in this study by recruiting large pregnancy cohorts of HR sibling fetuses, potentially collected across multiple research teams, and using imaging techniques (e.g., MRI) that are able to accurately measure third-trimester fetal brain growth.

In conclusion, the current study used a novel ultrasound method to explore cortical growth during the second-trimester in fetuses at increased risk for ASD. While we identified preliminary evidence for differences in growth trajectory between fetuses at HR and LR for ASD, there was no evidence that prenatal cortical measurements are predictive of later ASD. Due to the small sample size, these preliminary findings require replication in a much larger, independent cohort before we can confidently draw any definitive conclusions regarding prenatal brain growth in ASD.
References


Foreword to Chapters Four and Five

Chapters 2 and 3 reported on studies that examined prenatal neurodevelopment amongst infants at high- and low-risk for ASD. For two-dimensional ultrasounds, there were no significant differences between the groups in measurements of head circumference and body growth. However, utilizing a novel three-dimensional ultrasound technique revealed a unique pattern of cerebral volume growth during the second trimester amongst high-risk fetuses, relative to low-risk fetuses. This difference between groups was characterised by a slow rate of growth between 17 and 21 weeks’ gestation and a more rapid rate of growth from 22 weeks onwards in high-risk fetuses. However, individuals who were diagnosed with clinical ASD at age two years did not appear to contribute to this atypical growth trajectory. The preliminary data presented in these chapters identified several avenues for further research of prenatal markers of the ASD phenotype.

The thesis now turns to the second overarching aim, which was to understand when the behavioural differences between high- and low-risk infant siblings first emerge. Based on previous research, we obtained a broad range of behavioural observations including infant cry, parent-infant interaction, social-communication development and language ability during the first and second years of life in high- and low-risk infants. To address the second major aim, Chapter 4 reports on behavioural observations obtained at 6- and 12-months old compared between high- and low-risk infant siblings. The third and final aim was to examine, which of these behavioural characteristics of high-risk infants, if any, are predictive of later ASD outcomes at age two. Chapter 5 reports on the acoustic properties of infant cry data between 12-month old high- and low-risk infants. Chapter 4 and 5 present follow-up data for high-risk and low-risk siblings at two years old. We then examine whether behavioural observations and infant cry recordings obtained during the first year of life are predictive of ASD outcomes at age two.
CHAPTER 4  A longitudinal study of behavioural risk markers for Autism Spectrum Disorder in high risk infant siblings

This longitudinal study collected data from 31 infant siblings of children with Autism Spectrum Disorder (ASD) ('high risk’ group) and 42 infant siblings with no family history of ASD ('low risk’ group). At 6-months, one of the global ratings of parent-child interaction revealed differences between the groups, characterized by elevated negative affect in high-risk infants relative to low-risk infants (p < .05). At 12-month follow-up, high-risk and low-risk groups were differentiated on a standardized observation of ASD-risk but not on parent-report measures. By age two, high-risk infants were differentiated from low-risk infants on a broad range of standardized and informal assessments of ASD risk. Data collected at 12-months, but not at 6-months, were found to predict ASD/non-ASD classification at two years.

Despite research supporting a broad range of benefits to development for many, but not all children with ASD, who receive very early intervention (Helt et al., 2008), behavioral diagnoses of ASD are rarely confirmed before a child is three years old (Bent et al., 2015; Charman & Baird, 2002; Zwaigenbaum et al., 2009). Several longitudinal studies have sought to address this gap between time of symptom emergence and age of diagnosis by researching potential biological and behavioural risk markers for disorder during prenatal development and infancy (Hazlett et al., 2017; Unwin et al., 2016; Whitehouse et al., 2009). Prospective studies of siblings of ASD probands provide one methodology through which these risk markers in ASD can be investigated. Estimates of recurrence of ASD among siblings range between 10-20%, constituting a 20-fold increase in risk for the ASD phenotype (Ozonoff et al., 2011; Risch et al., 2014). Studies often involve the collection of data longitudinally, which can serve to develop our understanding of the impact of early delays and the emergence of ASD symptoms on long-term outcomes (Zwaigenbaum et al., 2007).
Previous prospective studies have identified several early emerging behaviours that differentiate ‘high risk’ (HR) infant siblings of ASD probands from ‘low risk’ (LR) infants with no family history of ASD. At 6-months old, HR infants have been differentiated from LR infants on a variety of behaviours including fewer vocalizations, a passive temperament (e.g. Zwaigenbaum et al., 2005), reduced social gaze, limited responsiveness to own name (Bryson et al., 2007) and unique neural response to eye gaze (Elsabbagh et al., 2012). Between 12 and 24-months old, the differences observed between HR and LR infants are more reliable, tend to be more pronounced and are predictive of ASD diagnosis at 24-36 month follow-up. For example, 12-month-old infants who are later diagnosed with ASD demonstrate less pointing and showing, fewer gestures (Barbaro & Dissanayake, 2013; Macari et al., 2012), reduced initiation of joint attention (Landa et al., 2007), poor imitation, fewer sensory-oriented behaviours, as well as delays in language, social communicative development and motor control (Dobkins et al., 2008; Veness et al., 2011), relative to typically developing infants (HR-no ASD and LR infants).

Nevertheless there is not a strong evidence base for behavioural markers for ASD in the first year of life, and current clinical guidelines recommend that these behaviours should not be used as early markers of the disorder (Zwaigenbaum et al., 2015). However, given that neuroplasticity is greatest during this early developmental period, which may maximise the effect of intervention, researchers continue to investigate potential behavioural markers prior to age one (Dawson, 2008). Observation of parent-child interaction provides another methodology for researching early disruptions in social-communication behaviours. Transactional models suggest that the presence of early delays in social skill development may influence how the caregiver responds to the interaction, which subsequently may contribute to further deficits in social functioning (Dawson, 2008; Elsabbagh & Johnson, 2010). Thus, according to the transactional model, it may be that specific characteristics of the parent-child dyad may indicate early disruptions in communication skills that are implicated in ASD trajectory (Green et al., 2015). Therefore, systematic observation of early parent-child interaction may be useful as a means of identifying signs of atypical social development prior to a diagnosis of ASD (Sameroff, 2009).

In a small sample of HR infants who were later diagnosed with ASD (HR-ASD; n = 15), Saint-Georges et al. (2011) examined retrospective video footage of parent-infant interaction and observed reduced social orientation and greater passivity in HR
infants, as well as increased directiveness among the HR infant’s caregivers, relative to LR infants. Early disruptions in social-communication have also been identified from prospective videos of parent-infant interaction (Campbell, Leezenbaum, Mahoney, Day, & Schmidt, 2015; Talbott, Nelson, & Tager-Flusberg, 2015; Wan et al., 2012, 2013; Yirmiya et al., 2006). For example, mothers of HR infants were more directive than LR mothers which was characterized by more frequent gesturing toward their infant during a play interaction, where maternal directiveness and gesture was associated with infant language ability and infant gesture use at 18-months old (Talbott et al., 2015). Further, Campbell et al. (2015) observed social reciprocity during free play between 11-month-old HR and LR infants and their caregivers, and found that HR-ASD infants were less engaged with their caregivers, relative to HR-no ASD and LR infants. At 11-months-old, social reciprocity score was predictive of later severity of ASD symptoms at 36-months old (Campbell et al., 2015).

Several recent studies of parent-infant interaction have used the Manchester Assessment of Caregiver-Infant Interaction (MACI; Wan et al., 2012) to characterize parent-child interactions. The MACI (Version 2) evaluates the quality of parent-infant interaction according to eight global rating scales. For example, ‘mutuality’ is one such scale and measures the dyadic togetherness and reciprocity in the play experience (Wan et al., 2012). Utilizing the MACI rating scheme, Wan et al. reported that 6-10 month-old HR infants were significantly less active, relative to LR infants and HR caregivers were more directive during play, used more gestures and were less sensitive in their pattern of responses toward their HR infant. Further, MACI ratings for the Dyadic Mutuality and Engagement scales have also revealed group differences between 12-month-old HR and LR infants and have predicted ADOS-G scores at follow-up (Wan et al., 2013). HR-ASD infants expressed less positive affect (or more negative) and paid less attention toward their caregivers compared to LR infants, but HR-no ASD were not significantly different from LR on ratings of attentiveness or affect (Wan et al., 2013).

The current study employed a prospective, longitudinal infant sibling design to further investigate early behavioural markers of ASD. Direct observations, standardized assessments and parent-reported behaviours of ASD risk were obtained for HR and LR groups when infants were 6-, 12- and 24-months old. The first aim in this study was to examine whether global MACI ratings of parent-infant interaction differentiated 6-month old HR and LR infants. The second aim was to determine whether standardized assessments and parent-report measures of ASD risk differentiated HR and LR groups
at 6-, 12- and 24-months old. The final aim was to evaluate whether any of the 6 or 12-month behavioural markers or interactional differences were predictive of a later diagnosis of ASD at age two. Given the body of research to date, it was hypothesized that behavioural markers would differentiate HR and LR siblings at 6- and 12-months old.

**Method**

**Participants**

The HR and LR infants and their families were part of the Pregnancy Investigation of Siblings and Mothers of children with autism (PRISM) study cohort in Perth, Western Australia. A total of 33 HR and 44 LR families were recruited to the study during the mother’s pregnancy via referrals from their obstetricians or gynaecologists or from local advertisements. For each family, enrolment in the PRISM study involved the collection of pre- and postnatal data over a period of three years. Two and three-dimensional fetal ultrasounds and umbilical cord blood were collected during prenatal and neonatal stages (Unwin et al., 2016), followed by a total of five postnatal follow-ups. A small proportion of families had relocated at the time of the follow-up assessments and were unable to participate through to the two-year outcome assessment. The final sample of participants, with two-year follow-up data, comprised 31 HR (17 male; 14 female) and 42 LR (19 male; 23 female) infants.

**Recruitment and assessment**

After enrolling in the PRISM study, families were invited to the Telethon Kids Institute and were asked to complete a detailed questionnaire related to their pregnancy and family history as well as participate in a face-to-face assessment. Parents completed the self-report Autism Quotient (AQ; Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001), the Edinburgh Postnatal Depression Scale (EPDS; Cox, Holden, & Sagovsky, 1987) and provided a self-reported history of mental health diagnoses. The AQ was used as a measure of autistic traits (where a score of 50 corresponded to more autistic traits) and the EPDS was used to identify mothers who may have been at-risk for perinatal depression, where scores above 13 on this measure are considered to be indicative of depression (Cox et al., 1987).
**Proband measures**

Probands in the HR group had previously received a clinical diagnosis of ASD according to Western Australia’s rigorous standard (Glasson et al., 2008). Probands were administered either the Mullen Scales of Early Learning (MSEL; Mullen, 1995) or Wechsler Intelligence Scale for Children-Fourth Edition (WISC-IV; Wechsler, 2003) depending on their age at time of assessment. The MSEL is a standardized assessment of cognitive ability that can be administered up to 68 months of age. The Early Learning Composite (ELC) score (calculated by combining T-scores across visual reception, language and motor domains) was used to compare groups on developmental ability. The WISC-IV is a standardized assessment of cognitive ability that can be used with children aged between 6 years and 16 years 11-months old. Composite and subtest scores can be calculated from the WISC-IV which are representative of intellectual functioning across a range of cognitive domains such as processing speed and working memory ability (Wechsler, 2003).

**PRISM study 6-month follow-up**

At the 6-month postnatal follow-up, HR and LR infant siblings and their caregivers participated in a 10 minute free play parent-infant interaction at their homes. Caregivers were instructed to play with their infant “as they normally would at home” using a set of standardized age-appropriate toys and the interaction was videotaped. The Manchester Assessment of Caregiver-Infant Interaction (MACI v2.1; Wan, 2016), was used to evaluate the quality of the interaction according to eight scales (on a 1-7 scale), including two caregiver scales (Sensitive Responsiveness and Non-Directiveness), four infant scales (Attentiveness to Parent, Positive Affect, Negative Affect and Liveliness), and two dyadic scales (Mutuality and Engagement Intensity). Six minute video clips were rated independently, with the coder blinded to HR/LR group membership. Interrater agreement was examined using intraclass correlation for 15 randomly selected recordings of parent-infant interaction. Disagreements between raters were resolved by re-reviewing the clips to reach consensus. Moderate to high agreement (all p < 0.001) was demonstrated for all MACI subscales: caregiver sensitive responsiveness, \( r = .66 \); caregiver non-directiveness, \( r = .80 \); infant attentiveness (to caregiver), \( r = .74 \); infant liveliness, \( r = .68 \), infant positive affect, \( r = .95 \); infant negative affect, \( r = .95 \); engagement intensity \( r = .63 \) and mutuality, \( r = .81 \).
At time of assessment, parents were also asked to complete the Communication and Symbolic Behavior Scales – Developmental Profile (CSBS DP; Wetherby & Prizant, 2002) and the Infant Behaviour Questionnaire-Revised very short form (IBQ-R; Putnam, Helbig, Gartstein, Rothbart, & Leerkes, 2014). The CSBS DP is a standardized 24-item parent-report checklist of social, communicative and symbolic abilities of children between 6- and 24-months of age. This tool has been validated as a first-level screener of ASD for children who obtain scores on the checklist that fall 1.25 SD below the mean (i.e. bottom 10th percentile of scorers; Pierce et al., 2011). The IBQ-R very short form is a 37-item parent-report measure of temperament traits for infants aged up to 18-months old (Gartstein & Putnam, 2012). The IBQ-R very short form can be scored according to three subscales, namely, Effortful Control, Negative Affect and Surgency (Gartstein & Putnam, 2012).

**PRISM study 12-month follow-up**

At the 12-month postnatal follow-up, caregivers completed the CSBS DP and infants were administered the MSEL (Mullen, 1995) and the Autism Detection in Early Childhood (ADEC; Young, 2007). The ADEC is comprised of 16-items and was administered to assess for the presence of any early ASD behavioural symptoms. A child’s score on this assessment can range from 0-32 and each item is give a score of 0-2 (where 0 corresponds to a typical response and scores of 1 or 2 correspond to an atypical response). A score of 11 or more is considered indicative of risk for ASD (Young et al., 2007).

**PRISM study two-year follow-up**

At the two-year postnatal follow-up, caregivers completed the CSBS DP and the HR/LR infant siblings were administered the MSEL, and the current ‘gold standard’ ASD assessment, the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000) module one. Infant siblings were then classified according to one of three criteria; Autism, Autism Spectrum or typical development. Standardized ADOS-G severity scores were calculated for the social affect (SA) and restricted and repetitive behaviours (RRB) domains (Hus, Gotham, & Lord, 2014). All three follow-up assessments were obtained within 3 weeks of the infant turning 6-, 12- or 24-months, respectively.
**Statistical Analysis**

Chi-square and one-way Analysis of Variance (ANOVA) were run to compare the characteristics of the family and proband demographics of the HR and LR groups. Using ANOVA, our analyses then turned to comparing HR and LR groups on mean MACI ratings of parent-infant interaction, CSBS DP, MSEL, IBQ, ADEC and ADOS-G severity at their respective follow-ups. If HR and LR groups differed on any potential confounding variables, analysis of covariance was used. Fisher’s exact test of independence was used to compare HR and LR groups on ADOS-G, ADEC and CSBS DP risk category, where the expected number of infants at-risk of ASD was small.

Pearson correlation analyses were then used to examine whether 6- and 12-month behavioural measures were correlated with two-year outcome measures. Finally, forced-entry multiple linear regression was used to predict ADOS-G scores (continuous) based on group membership (HR/LR) and 6- or 12-month measures.

**Results**

**Family demographics**

Proband and family characteristics are presented in Table 10. As expected, based on the higher incidence in ASD of males compared to females (Werling & Geschwind, 2013), 81% of the HR proband group were male, which compared with 43% of siblings in the LR group ($p < .001$). Results from one-way ANOVAs indicated that HR probands were significantly older at the time of their assessment ($p < .001$) and scored significantly lower on the MSEL ($p < .001$) compared to LR probands.
Table 10. *Family demographics for high- and low-risk groups. ‘High risk’ probands are siblings who are diagnosed with ASD, and ‘low risk’ probands are siblings who are typically developing.*

<table>
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<th>‘Low risk’ group (N = 42)</th>
<th>( \chi^2 )</th>
<th>( p )</th>
<th>( \phi )</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-35 years</td>
<td>9 (29)</td>
<td>20 (50)</td>
<td>3.18</td>
<td>.07</td>
<td>.21</td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>22 (71)</td>
<td>20 (50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>M (SD)</strong></td>
<td>M (SD)</td>
<td>( F )</td>
<td>( p )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proband age at assessment (years)</td>
<td>7.30 (5.39)</td>
<td>2.84 (2.67)</td>
<td>20.44</td>
<td>&lt;.01</td>
<td>1.05</td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite</td>
<td>78.16 (24.02)</td>
<td>106.34 (18.14)</td>
<td>24.85</td>
<td>&lt;.01</td>
<td>1.32</td>
</tr>
<tr>
<td>Wechsler Intelligence Scale for Children</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full scale IQ</td>
<td>80.79 (29.47)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maternal AQ</td>
<td>12.93 (6.35)</td>
<td>11.27 (4.55)</td>
<td>1.63</td>
<td>.21</td>
<td>.29</td>
</tr>
<tr>
<td>Paternal AQ</td>
<td>14.86 (7.10)</td>
<td>15.44 (6.36)</td>
<td>.12</td>
<td>.73</td>
<td>.09</td>
</tr>
</tbody>
</table>
Chi-square analyses identified no significant differences between the HR and LR groups in paternal age at conception, maternal and paternal mental health history and incidence of postnatal depression. There were also no significant differences between groups on maternal and paternal AQ scores. However, maternal age at conception was significantly older in the HR group, relative to the LR group (\(p < .05\)). Analyses of infant sibling demographics showed no significant differences between groups on sex or age at time of assessments (all \(p > .05\)).

**Between group comparisons at 6-month follow-up**

At 6-month follow-up, infant age was not significantly correlated with any of the MACI rating scales (all \(p > .05\)). Between-group differences for MACI global ratings were examined using one-way ANOVAs and are presented in Table 11. The HR group obtained significantly higher scores than the LR group on the Infant Negative Affect scale of the MACI (\(p < .05\)). While there was a trend towards between-group differences on the Infant Attentiveness and Dyadic Engagement Intensity MACI scales (HR infants showed less intense engagement with and attentiveness towards their caregiver), the differences were not significant (\(p < .08\) & \(p < .07\), respectively). Neither were there other statistically significant differences between groups on the remaining MACI scales. The HR group contained 7 of the 9 infants who obtained particularly high Infant Negative Affect scores (a rating of 5 or above). Further, one-way ANOVAs identified no significant differences between the HR and LR groups’ scores on the CSBS DP or the IBQ temperament scales (see Table 12 & Table 13).
Table 11. MACI global ratings of parent-infant interaction in HR (n = 28) and LR (n = 38) infant siblings

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group M (SD)</th>
<th>‘Low risk’ group M (SD)</th>
<th>F</th>
<th>p</th>
<th>η²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent sensitive responsiveness</td>
<td>3.32 (.98)</td>
<td>3.79 (1.26)</td>
<td>2.68</td>
<td>.11</td>
<td>.04</td>
</tr>
<tr>
<td>Parent non-directiveness</td>
<td>3.71 (1.38)</td>
<td>4.21 (1.34)</td>
<td>2.15</td>
<td>.15</td>
<td>.03</td>
</tr>
<tr>
<td>Infant attentiveness to parent</td>
<td>3.39 (1.23)</td>
<td>3.95 (1.23)</td>
<td>3.29</td>
<td>.08</td>
<td>.05</td>
</tr>
<tr>
<td>Infant positive affect</td>
<td>2.57 (1.60)</td>
<td>2.79 (1.60)</td>
<td>.30</td>
<td>.56</td>
<td>.01</td>
</tr>
<tr>
<td>Infant negative affect</td>
<td>3.04 (1.99)</td>
<td>2.08 (1.38)</td>
<td>5.31</td>
<td>.02</td>
<td>.08</td>
</tr>
<tr>
<td>Infant liveliness</td>
<td>4.18 (.98)</td>
<td>3.95 (1.06)</td>
<td>.81</td>
<td>.37</td>
<td>.01</td>
</tr>
<tr>
<td>Mutuality</td>
<td>2.86 (1.01)</td>
<td>3.29 (1.06)</td>
<td>2.11</td>
<td>.15</td>
<td>.03</td>
</tr>
<tr>
<td>Engagement intensity</td>
<td>3.39 (.96)</td>
<td>3.79 (.78)</td>
<td>3.45</td>
<td>.07</td>
<td>.05</td>
</tr>
</tbody>
</table>

### Between group comparisons at 12-month follow up

One-way ANOVA between the HR and LR groups identified no significant differences on the parent-report CSBS DP for either the total score or risk category. There was also no difference between HR and LR groups on the MSEL early learning composite (controlling for maternal age), or the expressive and receptive language subscale scores relative to LR infants. However, 6 (21%) of the HR group displayed moderate-risk behaviours on the ADEC compared to only 1 (2%) of the LR group (p < .05).

### Between group comparisons at two-year follow up

At two-year follow up, the HR group were differentiated from LR infants on a number of measures, including lower scores on the CSBS DP (total score and risk-category) and the MSEL, and higher ADOS-G severity scores. Six HR infants and one LR infant met ADOS-G criteria for ASD (p < .05).
Table 12. Characteristics of the high- and low-risk groups for categorical risk variables obtained at 6, 12 and 24-month follow up.

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group (N = 31)</th>
<th>‘Low risk’ group (N = 42)</th>
<th>p</th>
<th>φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-month ADOS-G classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism spectrum</td>
<td>6 (25)</td>
<td>1 (3)</td>
<td>.02</td>
<td>.34</td>
</tr>
<tr>
<td>Typical development</td>
<td>18 (75)</td>
<td>34 (97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-month CSBS DP</td>
<td></td>
<td></td>
<td>.04</td>
<td>.30</td>
</tr>
<tr>
<td>Concern</td>
<td>6 (23)</td>
<td>1 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No concern</td>
<td>20 (77)</td>
<td>32 (97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-month ADEC classification</td>
<td></td>
<td></td>
<td>.04</td>
<td>.30</td>
</tr>
<tr>
<td>Moderate-risk ASD</td>
<td>6 (21)</td>
<td>1 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-risk ASD</td>
<td>23 (79)</td>
<td>39 (98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-month CSBS DP</td>
<td></td>
<td></td>
<td>.21</td>
<td>.18</td>
</tr>
<tr>
<td>Concern</td>
<td>7 (29)</td>
<td>6 (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No concern</td>
<td>17 (71)</td>
<td>33 (85)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-month CSBS DP</td>
<td></td>
<td></td>
<td>.60</td>
<td>.10</td>
</tr>
<tr>
<td>Concern</td>
<td>10 (38)</td>
<td>12 (31)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No concern</td>
<td>16 (62)</td>
<td>27 (69)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 13. Characteristics of high- and low-risk groups for continuous variables at 6, 12 and 24-month follow up

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group (N = 31)</th>
<th>‘Low risk’ group (N = 42)</th>
<th>F</th>
<th>p</th>
<th>η²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6-month follow-up</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSBS DP Total</td>
<td>88.48 (17.04)</td>
<td>90.44 (16.29)</td>
<td>.11</td>
<td>.64</td>
<td>.00</td>
</tr>
<tr>
<td>Infant Behaviour Questionnaire-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effortful Control</td>
<td>4.99 (.70)</td>
<td>5.13 (.51)</td>
<td>.91</td>
<td>.35</td>
<td>.01</td>
</tr>
<tr>
<td>Negative Affect</td>
<td>4.04 (1.05)</td>
<td>3.64 (1.12)</td>
<td>2.08</td>
<td>.15</td>
<td>.03</td>
</tr>
<tr>
<td>Surgency</td>
<td>4.22 (1.13)</td>
<td>4.55 (.99)</td>
<td>1.57</td>
<td>.21</td>
<td>.02</td>
</tr>
<tr>
<td><strong>12-month follow-up</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite</td>
<td>102.14 (22.71)</td>
<td>105.85 (10.13)</td>
<td>.82</td>
<td>.37</td>
<td>.00</td>
</tr>
<tr>
<td>Receptive Language</td>
<td>49.41 (6.94)</td>
<td>51.80 (4.74)</td>
<td>2.88</td>
<td>.09</td>
<td>.04</td>
</tr>
<tr>
<td>Expressive Language</td>
<td>50.10 (7.98)</td>
<td>51.85 (6.09)</td>
<td>1.06</td>
<td>.31</td>
<td>.02</td>
</tr>
<tr>
<td>CSBS DP Total</td>
<td>91.56 (17.20)</td>
<td>96.38 (12.63)</td>
<td>1.29</td>
<td>.26</td>
<td>.02</td>
</tr>
<tr>
<td><strong>24-month follow-up</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite</td>
<td>105.31 (21.48)</td>
<td>119.26 (12.15)</td>
<td>10.34</td>
<td>.00</td>
<td>.15</td>
</tr>
<tr>
<td>Receptive Language</td>
<td>53.08 (13.39)</td>
<td>59.91 (6.31)</td>
<td>7.05</td>
<td>.01</td>
<td>.12</td>
</tr>
<tr>
<td>Expressive Language</td>
<td>52.00 (11.54)</td>
<td>62.89 (9.03)</td>
<td>17.10</td>
<td>.00</td>
<td>.22</td>
</tr>
<tr>
<td>CSBS DP Total</td>
<td>99.96 (20.55)</td>
<td>112.27 (15.54)</td>
<td>7.18</td>
<td>.01</td>
<td>.11</td>
</tr>
<tr>
<td>ADOS-G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social Affect Severity</td>
<td>2.75 (2.09)</td>
<td>1.69 (1.05)</td>
<td>6.66</td>
<td>.01</td>
<td>.10</td>
</tr>
<tr>
<td>RRB Severity</td>
<td>2.12 (2.34)</td>
<td>1.08 (.51)</td>
<td>6.48</td>
<td>.01</td>
<td>.10</td>
</tr>
</tbody>
</table>

**Relationship between early markers and two-year outcome**

Table 14 shows that none of the measures collected at 6-month follow-up were significantly correlated with behavioural measures collected at two-year outcome. However, at 12-months, the MSEL, CSBS DP and ADEC scores were all significantly
correlated with ADOS-G severity, CSBS DP and MSEL scores obtained at two-years old.

Table 14. Correlations between 6-month, 12-month and two-year follow-up data

<table>
<thead>
<tr>
<th></th>
<th>MACI Negative Affect</th>
<th>CSBS DP 6mths</th>
<th>Mullen ELC 12mths</th>
<th>ADEC 12mths</th>
<th>CSBS DP 12mths</th>
<th>Mullen ELC 12mths</th>
<th>CSBS DP two-years</th>
<th>ADOS-G RRB Severity</th>
<th>ADOS-G SA Severity</th>
<th>CSBS DP two-years</th>
<th>Mullen ELC two-years</th>
<th>ADEC 12mths</th>
<th>CSBS DP 12mths</th>
<th>Mullen ELC two-years</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADOS-G</td>
<td>-.10</td>
<td>.02</td>
<td>-.28**</td>
<td>.37**</td>
<td>-.34**</td>
<td>-.61**</td>
<td>-.55**</td>
<td>.58**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRB Severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADOS-G</td>
<td>.02</td>
<td>-.07</td>
<td>-.32**</td>
<td>.46**</td>
<td>-.41**</td>
<td>-.50**</td>
<td>-.43**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA Severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSBS DP two-years</td>
<td>.16</td>
<td>.09</td>
<td>.35**</td>
<td>-.48**</td>
<td>.55**</td>
<td>.52**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mullen ELC two-years</td>
<td>-.14</td>
<td>-.06</td>
<td>.46**</td>
<td>-.57**</td>
<td>.38**</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSBS DP 12mths</td>
<td>-.08</td>
<td>.36**</td>
<td>.43**</td>
<td>-.56**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADEC 12mths</td>
<td>-.11</td>
<td>-.21</td>
<td>-.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**p < .01, *p < .05

Three forced-entry multiple regressions were performed that included group membership (HR/LR) with each of the 12-month variables (MSEL early learning composite, CSBS DP and ADEC) as predictors of two year ADOS-G score (continuous). At 12-month follow-up, better performance on MSEL early learning composite ($B = -.08$, $F(1, 57) = 5.10$, $p < .05$) and CSBS DP ($B = -.09$, $F(1, 54) = 10.14$, $p < .01$) significantly predicted lower scores (less ASD symptomatology) on ADOS-G at two years old. Similarly, higher scores on the ADEC, collected at 12 months of age, were a significant predictor of higher scores on the ADOS-G ($B = .45$, $F(1, 58) = 8.58$, $p < .01$).
Discussion

Sibling cohorts provide one methodological platform to examine the emergence of risk factors for ASD prior to a diagnosis. Currently, there is an insufficient evidence base for the clinical use of any behavioural marker for ASD prior to 12-months of age (Zwaigenbaum et al., 2015). The first aim of this study was to investigate whether global MACI ratings of parent-infant interaction differentiated 6-month-old HR and LR infants. The second aim was to determine whether standardized assessments and parent-report measures of ASD risk differentiated HR and LR groups at 6-, 12- and 24-months old. The final aim was to evaluate whether any of the 6- or 12-month behavioural markers were predictive of later ASD classification according to ADOS-G at age two.

The current study replicated a previous study by Wan et al. (2012) that identified differences in global MACI ratings between HR and LR infants at 6-months of age. From direct observation of parent-child interaction, we observed that HR infants displayed more frequent and/or intense episodes of negative affect, relative to LR infants. In contrast, the two groups were not differentiated on parent-reported measures of social and communicative development. Furthermore, at 6-month follow-up, neither the parent-report nor detailed parent-infant interaction observations were predictive of ASD classification at two years old. At 12-month follow-up, performance on measures of developmental ability, social-communication and ASD risk were all predictive of scores on the ADOS-G (the current gold standard ASD outcome assessment) at age two. These observations provide further replication that formal and informal assessments of ASD risk and developmental milestones at age one, and not earlier, are predictive of later ASD symptomatology (Christensen et al., 2010; Ozonoff et al., 2010; Wan et al., 2013).

The current study also replicated the growing empirical research supporting differences between 12-month-old HR and LR infants (e.g. Dobkins et al., 2008; Macari et al., 2012; Veness et al., 2011; Zwaigenbaum et al., 2009). At this time, 21% of the HR group were identified at moderate-risk of ASD on the ADEC compared to 2% of the LR group. There was also a trend of lower scores on the MSEL, particularly for receptive language for HR infants but this difference was not statistically significant. At 12-months, HR infants did not obtain significantly different scores on parent-reported measures of social-communication development (i.e. CSBS DP), relative to the LR infants.
The current study found no evidence that behavioural measures obtained at 6-months old were predictive of ASD classification at age two. The increased negative infant affect observed during parent-child interaction in at-risk infants is consistent with a previous study that identified this temperament profile in a larger sample of two-year old HR siblings (with and without a later diagnosis of ASD, n = 138) (Garon et al., 2009). Our study indicates that affective differences may be captured far earlier when using a parent-infant interaction paradigm, which could be temperamentally related. Given that only a small number of HR siblings will develop ASD, HR infants differ from typically developing infants in the degree of negativity during social interaction well before signs of ASD are detected. Early delays in infant social skill development may influence how the parent responds to the interaction, which subsequently may contribute to further deficits in the child’s social functioning (Dawson, 2008; Elsabbagh & Johnson, 2010). Thus according to the transactional model it may be that specific characteristics of the parent-child dyad, such as infant affect, may serve to alert early disruptions in communication skills that are implicated in ASD trajectory or that subtle early disruptions in communication result in a more negative interaction (Green et al., 2015). For example, parents’ contribution to the unstructured play interaction may be perceived by the infant as less enjoyable and lead them to behave more negatively. Alternatively, a more stressful family environment with a child with ASD, may contribute to increased negativity among infant siblings. Ultimately, the affective difference between HR and LR infants may feature as part of the broader ASD phenotype.

Parent-mediated interventions designed to target early disruptions of the parent-child interaction have shown promising results. Green et al. (2015) trialed a parent-mediated intervention for infant probands and observed increases in infant attentiveness to their parent, reduced autism-risk behaviours, increased parental non-directiveness, improved attention disengagement and improved parent-rated infant adaptive function as outcomes of the intervention. These observations highlight the potential for early parent-child interaction markers to inform intervention and improve symptom trajectory in ASD.

We note several limitations of the current study design. First, the sample size under investigation was relatively small, which may have limited the statistical power to identify true effects. However, six (25%) of the HR infants were found to reach a clinical threshold for ASD on the ADOS-G at two-years of age, which is consistent with
previous estimates of risk for ASD among siblings of ASD probands. To maximise our statistical power, we examined 6- and 12-month behavioural variables in relation to continuous ADOS-G scores, though it is possible that a larger sample is still required to identify clear relationships over time. The current study was also limited by collecting only one recorded parent-infant interaction, though we note that our finding replicates previous studies (Wan et al., 2013).

Future research may consider examining parent-infant play behaviour across time in order to determine the trajectory of interaction behaviours in HR infant siblings. The strengths in this study lie in the comprehensive data collection inclusive of both formal testing, direct observation and parent-report which facilitated several comparisons of potential behavioural markers at the 6- and 12-month follow up.

In conclusion, the current study provides further support that observation of parent-child interaction prior to age one captures unique characteristics that differentiate HR infants from LR infants. While increased negative affect at 6-months in HR siblings did not predict ASD symptoms at age two, unique behavioural features from parent-infant interaction may represent suitable targets for early intervention. Preliminary studies of early intervention propose that optimising parent-infant interactions within the first 12-months of life can enhance developmental outcomes for infants at high risk of ASD (Green et al., 2015; Koegel, Singh, Koegel, Hollingsworth, & Bradshaw, 2013; Rogers et al., 2014; Steiner, Gengoux, Klin, & Chawarska, 2013). The current study also replicated the predictive value of 12-month behavioural markers for later ASD outcomes. Future research may consider investigating some of these behavioural risk indicators in a larger community risk sample. One such method involves recruiting infants who have been identified at-risk by a paediatrician or child health nurse following routine screening for communication delays or via self-referral. These cohorts may include a larger proportion of at-risk infants who receive a diagnosis of ASD at follow-up, which is required to facilitate in depth analyses of the relationship between early signs and later diagnosis.
References


Clifford, S., Hudry, K., Elsabbagh, M., Charman, T., Johnson, M. H., & Team, B.


Neurology, 44(5), 296–300.


CHAPTER 5  Acoustic properties of cries in 12-month old infants at high-risk of Autism Spectrum Disorder

There is preliminary evidence that infant siblings of children with Autism Spectrum Disorder (ASD) have an atypical pattern of cry, characterized by higher fundamental frequency and increased dysphonation. This prospective study collected multiple cry samples of 12-month old siblings of children with ASD (n = 22, ‘high-risk’ group) and 12-month olds with no family history of ASD (n = 27, ‘low risk’ group). While there was no difference between groups in the fundamental frequency or degree of phonation of the cry samples, the duration of each cry unit was significantly shorter in the high-risk siblings (p < .05). The six infant siblings who received a diagnosis of ASD at age two had amongst the shortest recorded cry durations. This study provides further evidence that atypical cry patterns may represent a very early manifestation of the ASD phenotype.

Crying is one of the first modes of communication between a human infant and their primary caregiver. Infants communicate their needs through their cry and rely on the caregiver’s responsiveness for survival. Compromised communication and social skills are one of the diagnostic criteria for Autism Spectrum Disorder (ASD; APA, 2013). This has led researchers to further examine the quality and phonetic properties of vocal production in young children diagnosed with ASD.

Several studies have observed atypical acoustic features in vocal productions of children with ASD (Esposito & Venuti, 2009; Oller et al., 2010; Schoen, Paul, & Chawarska, 2011; Sheinkopf, Mundy, Oller, & Steffens, 2000; Woods & Wetherby, 2003). For example, Esposito & Venuti (2009) analysed vocal productions from retrospective home videos obtained at approximately 12-months old for infants subsequently diagnosed with either ASD, developmental delay, or with no developmental disability. Infant crying behaviour and maternal responses were then
ACOUSTIC PROPERTIES OF CRY IN INFANTS AT RISK OF ASD

coded using an acoustic rating system called the Crying Observation Codes (Venuti et al., 2004). Infants with ASD were found to produce cry patterns characterized by increased dysphonation (shorter aspiratory/expiratory phase) and fewer pauses compared to the other two participant groups. It has been proposed that the underlying mechanism for these differences in cries of children with ASD may originate in neural abnormalities in the brainstem that disrupt the coordination of the larynx and the vocal tract during a cry episode (Rodier, 2002; Rodier, 1996). While these data may indicate a very early manifestation of ASD-related difficulties, the study design is limited by making use of retrospective video footage. The retrospective nature of the data results in several limitations, including the variability of the age of the participants, the difficulty in determining the developmental level or ASD symptomatology at the time of the recording and, finally, it can be challenging to control for the variability in length, content and structure in video footage.

Prospective designs are becoming increasingly common in the study of the early development of ASD. Prospective research in ASD typically involves the recruitment of infant sibling cohorts as a means of investigating potential risk factors longitudinally, prior to diagnostic age, where the recurrence risk of ASD in siblings is estimated to be 18.7% (Ozonoff et al., 2011). Sheinkopf, Iverson, Rinaldi & Laster (2012) prospectively examined the cries of a cohort of ‘high risk’ (HR) infant siblings of children with ASD (n = 17) and ‘low risk’ (LR) infants with no family history of ASD (n = 11). Cry samples were obtained when the infants were 6-months old, and were categorised as being either ‘pain’ or ‘non-pain’ related cries. The HR infants produced ‘pain-related’ cries that were significantly higher and more variable in fundamental frequency compared to the cries of LR infants (Sheinkopf et al., 2012). However, there were no significant differences between HR and LR infants in the acoustic characteristics cries categorized as ‘non-pain’ related cries. The infant cohort was followed until 36-months of age, at which time an ASD diagnostic assessment was performed. HR infants who were later diagnosed with ASD (HR-ASD), recorded the highest fundamental frequency for both types of cries (pain and non-pain) and their cries were significantly less phonated compared to typically developing infants (LR and HR-no ASD).

Prospective studies have since sought to replicate these findings. Observations of a higher fundamental frequency in cries of HR infants, relative to LR infants, has now been reported in several studies (Esposito & Venuti, 2010; Esposito, Carmen Rosagno, Venuti, Haltigan, & Messinger, 2014). Increased amplitude (Sheinkopf et al,
CHAPTER 5

2012), shorter duration (Esposito et al, 2014), increased dysphonation and a shorter length of pauses between cry episodes (Esposito & Venuti, 2010) have also been observed in HR infants during the first 12-months of life. Further, HR-ASD infants have been found to record among the highest values for fundamental frequency and produce poorly phonated cries relative to high- and low-risk infants who develop typically (Sheinkopf et al., 2012; Esposito et al., 2014; Esposito & Venuti, 2010). These studies have advanced this area by using the prospective research design. However, one limitation of these studies is relatively small samples of HR and LR infant siblings. For example, Sheinkopf et al. (2012) collected recordings from the sample of 17 HR and 11 LR infant siblings. After categorizing cries as either pain or non-pain related, the sample was reduced even further to seven HR infants and five LR infants for pain-related cries.

Previous research has also suggested that infants with ASD do not communicate their needs through crying as effectively as typically developing infants (Esposito & Venuti, 2008; Esposito et al., 2011; Venuti et al., 2012), making it more difficult for their parents to perceive what is causing them distress (Esposito & Venuti, 2008). For example, Venuti et al. (2012) utilized functional Magnetic Resonance Imaging to examine the brain activation patterns of a group of adults (n = 21) who were watching a series of retrospective video footage of infant cries. Whilst processing the cries of infants who were diagnosed with ASD, activity was more enhanced in brain regions associated with prosodic, emotional and verbal processing, relative to brain activation during processing of typically developing infant cries. Venuti et al. (2012) suggested that this may be due to unique acoustic properties of the cries (i.e. differences in fundamental frequency) that render them more difficult to interpret and that the increase in emotional activity. These cries may also be experienced more negatively by caregivers and found to be more emotionally arousing (Venuti et al., 2012). Taken together, these observations indicate that atypical acoustic properties of cries may represent a vocal signature of ASD in early life.

The aim in the present study was to examine the acoustic properties of multiple cries from a moderately sized sample of HR and LR infants at 12-months of age. Based on previous research, we hypothesized that this study would provide further support for an atypical acoustic profile in HR siblings of ASD. Specifically, we expected that cries from HR infants would be characterized by higher fundamental frequency, shorter cry durations and increased amplitude for the cry utterance. We then investigated whether
the acoustic properties of infant cries recorded at 12-months old were predictive of ASD symptomatology at two-years old.

**Method**

**Participants**

The HR and LR infants were part of the Pregnancy Investigation of Siblings and Mothers of children with autism (PRISM) study cohort in Perth, Western Australia. Two groups of families were recruited as part of this prospective, longitudinal study. The HR group comprised families who have at least one child with a diagnosis of ASD (the ‘proband’) and the LR group comprised families who have at least one child, older than three years, who has not received a diagnosis of any neurodevelopmental conditions.

A total of 33 HR and 44 LR families were recruited to the study during the mother’s pregnancy through referrals from their obstetricians or gynaecologists and via local newspaper and radio advertisements. For each family, enrolment in the PRISM study involved the collection of pre- and postnatal data over a period of three years. Two and three-dimensional fetal ultrasounds and umbilical cord blood were collected during prenatal and neonatal stages, followed by a total of five postnatal follow-ups (Unwin et al., 2016). Acoustic cry recordings were collected by parents at the 12-month follow up. A small proportion of families had relocated at the two year follow-up and were unable to participate. A subset of families participated in the 12-month home visit with a researcher, but due to their other commitments they did not collect acoustic recordings in the weeks following the visit. The final sample of available acoustic recordings was 23 HR and 33 LR infants.

**Recruitment and assessment**

Families were invited to the Telethon Kids Institute (University of Western Australia) and were asked to complete a detailed questionnaire related to their health, demographics, pregnancy and family history. Caregivers were also asked to provide details relating to the early behavioural development of the proband child.
Probands in the HR group were administered the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000) and all but four of the probands met criteria for ASD. Based on the rigorous diagnostic practices mandated in Western Australia (i.e. consensus by a team of clinicians, Glasson et al., 2008), a decision was made to include all participants in further analyses. In all four cases, study investigators cited the original diagnostic report and confirmed the diagnosis of ASD. Proband children were also administered the Mullen Scales of Early Learning (MSEL; Mullen, 1995) to provide a standardized assessment of cognitive ability. Early Learning Composite (ELC) scores (based on ability in the areas of visual reception, language and motor skills) are reported in the Results section. Probands participated in either the MSEL, the Wechsler Intelligence Scale for Children (WISC-IV; Wechsler, 2003) or the Wechsler Abbreviated Scale of Intelligence (WASI; Wechsler, 1999) assessments, depending on their age at the time of assessment.

**PRISM study 12-month follow-up**

At the 12-month postnatal follow-up, the HR and LR infant siblings completed the MSEL and the Autism Detection in Early Childhood (ADEC; Young, 2007) to assess for early developmental delay and for the presence of any early ASD behavioural symptoms, respectively. The ADEC tool is comprised of 16 items; each item is given a score ranging between 0-2 (0 = typical response, 1-2 = inappropriate response). Total scores can range from 0-32, where a score of 11 or more is considered indicative of risk for ASD (Young, 2007).

Following the 12-month postnatal follow-up, parents were provided with a Sony 2GB icd-px312 voice recorder and were asked to record samples of their infant crying. All cry recordings were saved in mp3 format. Cries were to be as naturalistic as possible with recorded cries occurring spontaneously rather than being elicited by the carer. Parents were asked to hold the voice recorder as close to the child as possible in order to minimize surrounding sounds. Infant positioning was not manipulated; however, once a recording had been captured, parents were asked to note on each occasion whether their child was sitting, standing or lying down, since this may have impacted on the quality of the recording (Lin & Green, 2007) and also to comment on the perceived reason for the cry. Parents were given the recorder for a period of 1-2 weeks and were encouraged to record at least one sample of infant cry during the daytime and one during the evening.
All recordings were collected by parents within 4 weeks of the infant siblings’ first birthday.

**Acoustic analysis procedures**

Acoustic analyses were performed using Praat voice analysis software (Boersma & Weenink, 2005) by an independent researcher who was naïve to group membership. The signal was low pass filtered at 10,000 Hz and the sampling rate was 44,100 Hz (Rautava et al. 2007). A total of 247 mp3 formatted cry recordings were collected by parents and for each infant, at least one cry episode was obtained. Cry episodes were often terminated at points where parents intervened and comforted their child. Thus, for acoustic analysis, cry units were extracted from the episodes of crying. A cry unit was defined as the expiratory phase of respiration during a cry which lasts a minimum of 0.5 seconds (Sheinkopf et al., 2012). All valid cry units were extracted from the episode of crying that was recorded for each infant. For each infant cry episode the aim was to extract and analyse three cry units.

A total of 146 cry units (three per participant with the exception of one participant with only two acceptable cry units) were extracted and identified as suitable for acoustic analysis when background noise was minimal to avoid interference in the analysis. Recordings with less than 30dB difference between the mean intensity of the cry episode and the mean intensity of the nearest pause were excluded from the study, as per the guidelines proposed by Deliyski et al. (2005). Where the difference in intensity was less than 30dB, cry units were extracted from the next recording and reanalysed for differences in intensity until each child had three acceptable cry units. If three units of acceptable quality could not be extracted from the same recording, units were extracted from multiple recordings.

Only voiced segments were included in analyses in order to prevent interference from non-cry characteristics, such as coughs and pauses during expiration. Extracting the voiced segments required identifying the voice boundaries within the cry unit. Boundaries were distinguished through spectrographic analysis focusing on waveform contours, pitch lines and the onset and conclusion of pulses. Segments that were void of pulses and pitch lines indicate the absence of phonation and were therefore excluded from analysis. Voiced segments were extracted and then concatenated to produce an exclusively voiced cry unit for calculating acoustic measures.
Dependent measures produced by acoustic analyses were fundamental frequency (F0), the amplitude, formant frequencies (F1, F2), and cry duration. The mean fundamental frequency of the voiced segment was calculated with the parameters set to 100 Hz for the pitch floor and 1000 Hz for the pitch ceiling. These boundaries were selected based on previous evidence indicating that spontaneous infant cries range from 200Hz to 600Hz (e.g. Etz, 2014; Michelsson, Eklund, Leppanen & Lyytinen, 2002). The minimum and maximum F0 was obtained to allow for observations in the variance in F0. As intensity scores can reflect neural control and the capacity of the respiratory system (LaGasse et al., 2005), the minimum, maximum and range of intensity values were recorded. The first and second formants (F1 and F2) were calculated as they relate to the F0 and reflect vocal tract control (Santos et al., 2013). Finally, the cry duration was documented after being extracted from the original recording as the expiratory phase of respiration lasting a minimum of 0.5 seconds. Coughs and pauses were included when originally segmenting the cries from the sample, but then excluded from analyses for all variables except cry duration. The cry was determined to have ended at the time of inhale. For each cry, acoustic parameters were computed with Praat software. Definitions of acoustic variables and their corresponding biological mechanisms are described in Table 15.

A total of 56 families collected voice recordings for HR (n = 23) and LR (n = 33) infant siblings. Recordings from seven participants were excluded as they either did not satisfy the established requirements for sound quality (the difference between the mean intensity of the cry episode and the mean intensity of the nearest pause was less than 30dB) or the files were not originally saved in, and could not be converted to mp3 format (n = 3) due to the method of collection (i.e. a parent who attempted to collect extra recordings on their mobile phone device). For one participant, there were only two (instead of three) cry units that met criteria for acoustic analysis. In an effort to maximize the sample size, we decided to include this participant in further analyses. The final sample size consisted of 22 HR and 27 LR infant siblings (a total of 146 cry units).

**Coding of Cry Episodes**

Additional coding was performed to detail the parent-reported cause of infant distress (e.g. hunger, fatigue, frustration) and researcher’s perceived level of infant distress. To obtain a cry distress rating, one researcher who was blind to the group
assignment listened to one cry recording for each infant and rated their perception of the infant’s distress. Adapted from Esposito et al. (2015), perception of infant distress was recorded using a 7-point Likert scale, where a score of 1 corresponded to lowest level of distress and a score of 7 corresponded to the highest level of distress.

**PRISM study two-year follow-up**

At the two-year postnatal follow-up, the HR and LR infant siblings were administered the MSEL, and the current ‘gold standard’ ASD assessment, the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000) module one. Infant siblings were then classified according to one of two criteria; Autism Spectrum or none. Standardized ADOS-G severity scores were calculated for the social affect (SA) and restricted and repetitive behaviours (RRB) domains (Hus et al., 2014). All follow-up assessments were obtained within 3 weeks of the infant turning 12- or 24-months, respectively.
Table 15. Description and biological mechanism for each of the acoustic variables.

<table>
<thead>
<tr>
<th>Acoustic variables</th>
<th>Definition</th>
<th>Biological mechanism</th>
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<tbody>
<tr>
<td><strong>Fundamental frequency (F0)</strong></td>
<td>The fundamental frequency was computed using the Praat autocorrelation algorithm (Boersma &amp; Weenink, 2005). The algorithm was parameterized to use a Gaussian window. The mean fundamental frequency of the voiced segment was calculated with the parameters set to 100 Hz as the pitch floor and 1000 Hz as the pitch ceiling. The settings were selected as spontaneous infant cries range from 200Hz to 600Hz and have been set as such in similar studies (Etz et al., 2014). The mean F0 was calculated across three cry units. The minimum and maximum fundamental frequency was also obtained to calculate the variance in F0. The fundamental frequency is perceived as the pitch of the cry.</td>
<td>Number of glottal openings per second. Control of the lower vocal tract will be reflected by variations in pitch (including instability and deviations in maximum and minimum values).</td>
</tr>
<tr>
<td><strong>Amplitude</strong></td>
<td>Intensity of the cry (dB). Heard as loudness. The minimum and maximum intensity values were obtained as was the range of intensity. Constant noise levels that might have been introduced by the microphone were subtracted by Praat’s intensity algorithm automatically. In addition, the algorithm filters pitch-synchronous intensity variations (Boersma &amp; Weenink, 2013).</td>
<td>Neural control, and capacity, of the respiratory system and will be reflected in abnormalities in loudness of the cry (including deviations in maximum and minimum values and instability).</td>
</tr>
<tr>
<td><strong>First and second formants (F1, F2)</strong></td>
<td>Frequencies centred at first and second resonance of F0. Formant ceiling was set to 8000 Hz which is in line with prior research methods (Etz et al., 2014). The mean F1 and F2 was calculated in Praat across three cry units.</td>
<td>Neural control of the size and shape of the upper vocal tract, which will be reflected in changes to the resonance characteristics of the cry.</td>
</tr>
<tr>
<td><strong>Cry duration</strong></td>
<td>The length of cry units measured in seconds was documented after being extracted from the original recording. Boundaries of cries were identified by spectrographic analysis, based on the intensity contour as well as the waveform and the spectrum. Cry duration was then computed as the duration of the extracted cry. This measure was documented before excluding non-voiced segments and therefore includes coughs and minor pauses.</td>
<td>Neural control of the respiratory system, which will be reflected in changes to the duration and pause characteristics of the cry.</td>
</tr>
</tbody>
</table>
ACOUSTIC PROPERTIES OF CRY IN INFANTS AT RISK OF ASD

Statistical Analysis

Characteristics of the probands and family demographics of the HR and LR groups were compared using chi-square and one-way Analysis of Variance (ANOVA). Prior to performing group analyses for acoustic variables, medians were calculated for each dependent variable across the three cry episodes. The median statistic was selected as a measure of central tendency to reduce the influence of variability, especially extreme scores, across the three episodes per infant, whilst still providing detailed information about each participant’s cry. For the one participant with only two cry episodes, a mean statistic was calculated for each dependent variable. Using ANOVA, our analyses then turned to comparing HR and LR groups on F0 (min, max, variance), F1, F2, the amplitude (min, max, variance), and cry duration. Groups were also compared on differences in performance for MSEL, ADEC and ADOS-G severity at their respective follow-ups. If HR and LR groups differed on any potential confounding variables, analysis of covariance was used. Fisher’s exact test of independence was used to compare HR and LR groups when the expected number of infants was small. Pearson correlation analyses were then used to examine whether 12-month acoustic measures were correlated with two-year outcome measures.

Results

Proband characteristics are presented in Table 16 and family demographics for the HR and LR infant data are reported in Table 17. As expected, based on the higher incidence in ASD of males compared to females (Werling & Geschwind, 2013), 79% of the HR proband group were male, which compared with 48% of siblings in the LR group ($p = .01$). Results from one-way ANOVAs indicate that HR probands were significantly older at the time of their assessment ($p < .05$) and scored significantly lower on the MSEL ($p < .001$) compared to LR probands.

Fisher’s exact test of independence identified no significant differences between the HR and LR groups in maternal smoking, paternal age at conception or maternal alcohol use during pregnancy. However, maternal age at conception was significantly older in the HR group ($p < .05$), compared to the LR group. Analyses of infant sibling demographics observed no significant differences between groups on sex, gestational
age at birth, age at the time of assessment, nor in the average number of acoustic recordings collected by the parent(s). Family size was not significantly different between the HR and LR groups. There was also no statistically significant difference between groups in the proportion of HR (14%) and LR (0%) infants that were born preterm (prior to 37 weeks gestation, \( p > .05 \)).

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group (N = 22)</th>
<th>‘Low risk’ group (N = 27)</th>
<th>( p )</th>
<th>( \phi )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal smoking during pregnancy</td>
<td>1 (4.5)</td>
<td>0 (0)</td>
<td>.45</td>
<td>.17</td>
</tr>
<tr>
<td>Maternal alcohol intake at least weekly during pregnancy</td>
<td>1 (4.5)</td>
<td>1 (3.7)</td>
<td>1.00</td>
<td>.03</td>
</tr>
<tr>
<td>Maternal age at conception</td>
<td></td>
<td></td>
<td>.04</td>
<td>.32</td>
</tr>
<tr>
<td>20-35 years</td>
<td>5 (23)</td>
<td>15 (56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>17 (77)</td>
<td>12 (44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paternal age at conception</td>
<td></td>
<td></td>
<td>.23</td>
<td>.18</td>
</tr>
<tr>
<td>20-35 years</td>
<td>5 (23)</td>
<td>11 (41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>17 (77)</td>
<td>16 (59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibling sex</td>
<td></td>
<td></td>
<td>.78</td>
<td>-.04</td>
</tr>
<tr>
<td>Male</td>
<td>12 (55)</td>
<td>13 (48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10 (45)</td>
<td>14 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibling preterm births</td>
<td></td>
<td></td>
<td>.07</td>
<td>.31</td>
</tr>
<tr>
<td>M (SD)</td>
<td>37.90 (1.62)</td>
<td>38.65 (1.09)</td>
<td>3.55</td>
<td>.07</td>
</tr>
<tr>
<td>Sibling gestational Age at birth (weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibling age at assessment (months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N of cry recordings</td>
<td>5.14 (3.78)</td>
<td>4.56 (2.61)</td>
<td>.40</td>
<td>.53</td>
</tr>
<tr>
<td>N of other siblings</td>
<td>1.37 (1.16)</td>
<td>1.11 (.42)</td>
<td>1.12</td>
<td>.30</td>
</tr>
</tbody>
</table>
Table 16. Characteristics of probands in the two groups. 'High risk' probands are siblings who are diagnosed with ASD and 'low risk' probands are siblings who are typically developing.

<table>
<thead>
<tr>
<th></th>
<th>'High risk' group (N = 22)</th>
<th>'Low risk' group (N = 27)</th>
<th>p</th>
<th>φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
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<td></td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td>.01</td>
<td>-.37</td>
</tr>
<tr>
<td>Male</td>
<td>18 (83)</td>
<td>12 (44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4 (17)</td>
<td>15 (56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADOS classification</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Autism</td>
<td>15 (68.2)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism spectrum</td>
<td>3 (13.6)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4 (18.2)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at assessment (years)</td>
<td>7.60 (6.06)</td>
<td>3.10 (3.21)</td>
<td>10.51</td>
<td>.00</td>
</tr>
<tr>
<td>M (SD)</td>
<td>M (SD)</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td>n = 14</td>
<td>n = 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite</td>
<td>75.07 (26.53)</td>
<td>108.00 (17.86)</td>
<td>21.42</td>
<td>.00</td>
</tr>
<tr>
<td>Wechsler Intelligence Scale for Children</td>
<td>n = 7</td>
<td>n = 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full scale IQ</td>
<td>89.50 (31.39)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wechsler Abbreviated Scale for Intelligence</td>
<td>n = 0</td>
<td>n = 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full scale IQ</td>
<td>-</td>
<td>105</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Between-group comparisons at 12-month follow-up

Analyses identified no significant differences between groups for the F0, F1, F2, or any of the amplitude measurements (Error! Reference source not found.). However, cry duration was significantly shorter for the HR group relative to the LR group ($p < .05, \eta^2 = .08$). One-way ANOVA between the HR and LR groups identified significantly poorer performance on the MSEL composite score ($p < .05, \eta^2 = .12$) and elevated risk scores on the ADEC ($p < .05, \eta^2 = .16$) in the HR group relative to the LR group. At 12-month follow-up, there were no significant differences between the HR and LR groups on MSEL receptive or expressive language scores.

Four participants in the HR group (18.1%) and one participant in the LR group (3.7%) scored above the risk threshold falling within the moderate-risk range on the ADEC (Table 19). Participants who scored above the ADEC risk threshold appeared to have the shortest cry durations. That is, infants with scores in the moderate-risk range ($n = 5$) obtained a maximum cry duration of 2.23 seconds compared to 4.76 seconds for infants scoring in the low-risk range ($n = 44$). Cry duration was not significantly correlated with ADEC scores for the total sample. Removing the HR infant siblings of the four probands who did not meet criteria for ASD on the ADOS-G did not change the pattern of effects observed.
Table 17. Characteristics of the high- and low-risk infant groups

<table>
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<tr>
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<tr>
<td>pregnancy</td>
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<td></td>
</tr>
<tr>
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<td>1 (4.5)</td>
<td>1 (3.7)</td>
<td>1.00</td>
<td>.03</td>
</tr>
<tr>
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<tr>
<td>pregnancy</td>
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<td></td>
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<td></td>
<td></td>
<td>.23</td>
<td>.18</td>
</tr>
<tr>
<td>20-35 years</td>
<td>5 (23)</td>
<td>11 (41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;35 years</td>
<td>17 (77)</td>
<td>16 (59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibling sex</td>
<td></td>
<td></td>
<td>.78</td>
<td>-.04</td>
</tr>
<tr>
<td>Male</td>
<td>12 (55)</td>
<td>13 (48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10 (45)</td>
<td>14 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibling preterm births</td>
<td>3 (14)</td>
<td>0 (0)</td>
<td>.07</td>
<td>.31</td>
</tr>
<tr>
<td>M (SD)</td>
<td>37.90 (1.62)</td>
<td>38.65 (1.09)</td>
<td>.55</td>
<td>.07</td>
</tr>
<tr>
<td>Sibling gestational Age at</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>birth (weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibling age at assessment</td>
<td></td>
<td></td>
<td>.95</td>
<td>.33</td>
</tr>
<tr>
<td>(months)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N of cry recordings</td>
<td>5.14 (3.78)</td>
<td>4.56 (2.61)</td>
<td>.40</td>
<td>.53</td>
</tr>
<tr>
<td>N of other siblings</td>
<td>1.37 (1.16)</td>
<td>1.11 (.42)</td>
<td>1.12</td>
<td>.30</td>
</tr>
</tbody>
</table>
Table 18. One-way Analysis of Variance between high- and low-risk siblings for the acoustic measurements.

<table>
<thead>
<tr>
<th>Acoustic variables</th>
<th>High-risk group (n = 22)</th>
<th>Low-risk group (n = 27)</th>
<th>F</th>
<th>p</th>
<th>η²</th>
</tr>
</thead>
<tbody>
<tr>
<td>M (SD)</td>
<td>M (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F0 (Hz)</td>
<td>420.91 (65.48)</td>
<td>444.11 (88.65)</td>
<td>.01</td>
<td>.92</td>
<td>.00</td>
</tr>
<tr>
<td>Min F0 (Hz)</td>
<td>248.14 (87.45)</td>
<td>259.86 (80.99)</td>
<td>.27</td>
<td>.61</td>
<td>.01</td>
</tr>
<tr>
<td>Max F0 (Hz)</td>
<td>706.66 (179.56)</td>
<td>697.29 (160.16)</td>
<td>.00</td>
<td>.99</td>
<td>.00</td>
</tr>
<tr>
<td>Variance F0 (Hz)</td>
<td>432.80 (205.78)</td>
<td>437.98 (187.22)</td>
<td>1.40</td>
<td>.71</td>
<td>.00</td>
</tr>
<tr>
<td>Min Amplitude (dB)</td>
<td>69.68 (5.43)</td>
<td>68.19 (6.12)</td>
<td>1.27</td>
<td>.27</td>
<td>.03</td>
</tr>
<tr>
<td>Max Amplitude (dB)</td>
<td>87.04 (.90)</td>
<td>86.84 (1.35)</td>
<td>.56</td>
<td>.45</td>
<td>.01</td>
</tr>
<tr>
<td>Variance Amplitude (dB)</td>
<td>17.18 (4.84)</td>
<td>18.35 (6.41)</td>
<td>.58</td>
<td>.45</td>
<td>.01</td>
</tr>
<tr>
<td>F1 (Hz)</td>
<td>1121.10 (308.82)</td>
<td>1050.84 (240.81)</td>
<td>.95</td>
<td>.33</td>
<td>.02</td>
</tr>
<tr>
<td>F2 (Hz)</td>
<td>2370.10 (224.47)</td>
<td>2368.47 (228.80)</td>
<td>.02</td>
<td>.89</td>
<td>.00</td>
</tr>
<tr>
<td>Cry duration (seconds)</td>
<td>1.58 (.54)</td>
<td>2.03 (.82)</td>
<td>4.43</td>
<td>.04</td>
<td>.08</td>
</tr>
</tbody>
</table>

A non-parametric independent-samples t-test was performed to compare the perceived level of distress ratings between the HR and LR groups. The difference between groups on researcher ratings of perceived level of distress was not significant (Mann-Whitney U = 270, p > .05).

**Between-group comparisons at two-year follow up**

At two-year follow up, the HR group were differentiated from LR infants on a number of measures, including lower scores on the MSEL, and higher ADOS-G severity scores. Five HR infants and one LR infant met ADOS-G criteria for ASD (p < .05) (Table 19).
Table 19. Characteristics of high- and low-risk sibling groups for continuous and categorical variables at 12 and 24-month follow up.

<table>
<thead>
<tr>
<th></th>
<th>‘High risk’ group (N = 22)</th>
<th>‘Low risk’ group (N = 27)</th>
<th>F</th>
<th>p</th>
<th>(\eta^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12-month follow-up</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td>n = 22</td>
<td>n = 27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite</td>
<td>97.76 (11.28)</td>
<td>105.22 (10.45)</td>
<td>6.13</td>
<td>.02</td>
<td>.12</td>
</tr>
<tr>
<td>Receptive Language</td>
<td>49.55 (7.45)</td>
<td>51.93 (4.72)</td>
<td>2.21</td>
<td>.14</td>
<td>.05</td>
</tr>
<tr>
<td>Expressive Language</td>
<td>49.73 (8.87)</td>
<td>51.41 (6.13)</td>
<td>.87</td>
<td>.36</td>
<td>.02</td>
</tr>
<tr>
<td>ADEC Total score</td>
<td>7.59 (3.13)</td>
<td>5.44 (2.17)</td>
<td>8.72</td>
<td>.01</td>
<td>.16</td>
</tr>
<tr>
<td><strong>Two-year follow-up</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mullen Scales of Early Learning</td>
<td>n = 22</td>
<td>n = 25*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite</td>
<td>104.55 (20.90)</td>
<td>117.28 (12.63)</td>
<td>6.76</td>
<td>.01</td>
<td>.13</td>
</tr>
<tr>
<td>Receptive Language</td>
<td>53.00 (13.48)</td>
<td>58.96 (6.27)</td>
<td>3.69</td>
<td>.05</td>
<td>.08</td>
</tr>
<tr>
<td>Expressive Language</td>
<td>51.82 (11.52)</td>
<td>61.88 (9.24)</td>
<td>10.75</td>
<td>.00</td>
<td>.20</td>
</tr>
<tr>
<td>ADOS-G</td>
<td>n = 22</td>
<td>n = 25*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Score</td>
<td>3.71 (4.06)</td>
<td>1.60 (2.12)</td>
<td>5.31</td>
<td>.03</td>
<td>.11</td>
</tr>
<tr>
<td>Social Affect Severity</td>
<td>2.71 (2.08)</td>
<td>1.64 (1.11)</td>
<td>5.10</td>
<td>.03</td>
<td>.11</td>
</tr>
<tr>
<td>RRB Severity</td>
<td>2.05 (2.33)</td>
<td>1.00 (.00)</td>
<td>5.36</td>
<td>.03</td>
<td>.11</td>
</tr>
<tr>
<td>24-month ADOS-G classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autism Spectrum</td>
<td>5 (24)</td>
<td>1 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>16 (76)</td>
<td>24 (96)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-month ADEC classification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate-risk ASD</td>
<td>4 (18)</td>
<td>1 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-risk ASD</td>
<td>18 (82)</td>
<td>26 (96)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Relationship between early markers and two year outcome

Pearson correlation analyses revealed a trend towards significance for shorter cry durations correlating with more severe ADOS-G restricted and repetitive behaviours severity score ($p = .08$) and poorer performance on MSEL receptive language ($p = .07$) and MSEL receptive language performance ($p = .06$). The infant siblings who received a diagnosis of ASD at age two ($n = 6$) had amongst the shortest recorded cry durations with a maximum cry duration of 2.72 seconds compared to a maximum duration of 3.80 seconds for infants who did not receive a diagnosis of ASD ($n = 43$). Length of phonation by ADOS-G diagnosis (ASD or none) and risk group (HR or LR) are presented in Figure 3.

![Figure 3](image)

*Figure 3. Length of phonation by risk group and ADOS-G diagnostic classification.*

Blue dots correspond to infants who did not receive a diagnosis of ASD and black dots correspond to infants who did receive a diagnosis of ASD.


**Discussion**

Previous research has observed atypical patterns in the acoustic properties of cries in at-risk infants, irrespective of whether they are later diagnosed with ASD. These studies have often relied on small participant numbers and have employed a retrospective study design, making it difficult to account for confounding variables. The present study aimed to overcome these previous limitations by prospectively analysing the acoustic properties of multiple cries of 12-month old HR and LR siblings enrolled in the PRISM study cohort in Western Australia. We hypothesized that this research would provide further support for an atypical acoustic profile characterized by higher fundamental frequency, shorter cry durations and an increased amplitude in HR relative to LR siblings. We also investigated whether acoustic properties of infant cries recorded at 12-months old were predictive of ASD symptomatology at two years old.

The hypothesis was only partially supported, with cry duration found to be significantly shorter for the HR group relative to the LR group. Of this sample, the infant siblings who obtained elevated scores on ASD-risk measurements at 12-months of age had amongst the shortest cry durations recorded. These findings are comparable with previous research that observed infants subsequently diagnosed with ASD to show a smaller proportion of the cry sequence occupied by the aspiration/expiration phase compared to infants subsequently identified as either typically developing or developmentally delayed (Esposito & Venuti, 2009). Furthermore, shorter cry durations have been previously recorded amongst 15-month old HR infants relative to LR infants, and of these, those toddlers who were diagnosed with ASD (HR-ASD), recorded the shortest cry durations of the sample (Esposito et al., 2014).

Neural control of the respiratory system is thought to be the biological mechanism underlying shorter cry duration, where shorter utterances are indicative of increased instability and tension of neural control of the vocal tract and poorer capacity and control of the respiratory system (LaGassee et al., 2005). The same brainstem structures are involved in both vocalizations via the pharyngeal and laryngeal muscles and the regulation of heart rate via the vagus (Stewart et al., 2013). Subsequently, shorter cry durations have also been linked to disruptions in autonomic regulation (i.e. increased heart rate) (Steward et al., 2013). Research into neurological characteristics of
ASD has described abnormalities within the brainstem, possibly due to complications at the time of neural tube closure (Rodier, 2002). Specific areas of abnormalities identified vary between studies including shortening of the midbrain, a major reduction of neurons in the facial nucleus (Bailey et al. 1998), and an enlarged arcuate nuclei in the medulla which is involved in respiratory regulation (Rodier, 1996). An individual’s inspiration and expiration capacity can be influenced by instability in neural control of the respiratory system (Stewart et al., 2013). In the case of infant cries, it can be reflected in the length and amplitude of expiration which has been shown to be shorter and louder in some children with ASD (Esposito et al, 2014; Sheinkopf, Iverson, Rinaldi, & Lester et al, 2012). The variability of findings makes it difficult to pinpoint an exact area of deficit in individuals with ASD, however there does appear to be evidence to support brainstem malformation.

At two-year follow-up, the HR group was differentiated from LR infants on a number of measures, including lower scores on the MSEL (indicative of poorer performance), and higher ADOS-G severity scores (indicative of elevated ASD-risk). Six infants who met ADOS-G criteria for ASD at two-years old, recorded some of the shortest cry durations at 12-months old. There was a trend toward significance for shorter cry durations to be associated with more severe ADOS-G restricted and repetitive behaviours severity score and poorer performance on the MSEL language subscales. These results provide some preliminary evidence for a unique acoustic profile characterized by the shorter overall duration of cries in infants at-risk of ASD. Further research is required to better understand the biological and physiological underpinnings of this finding in the context of ASD. Future research may consider investigating some of these behavioural risk indicators in a larger community risk sample. These cohorts may include a larger proportion of at-risk infants who receive a diagnosis of ASD at follow-up, which is required to facilitate in depth analyses of the relationship between early signs and later diagnosis.

There were no significant differences between the HR and LR infants in measures of fundamental frequency, first and second formants or the amplitude of cries. These observations are inconsistent with previous research that has reported higher fundamental frequency measurements for HR infants, relative to LR infants (e.g. Sheinkopf et al., 2012; Esposito et al., 2014). The Sheinkopf et al. (2012) study, which categorized cries as pain-related or non-pain related, observed the elevation in F0 for HR infants (HR-ASD and HR-no ASD) in the pain-related cries only. Esposito et al.
(2014) similarly observed elevations in fundamental frequency among 15-month old HR when compared to LR toddlers. In their study, cries were elicited during a social attachment paradigm known as the Strange Situation (Ainsworth and Wittig, 1969; Ainsworth et al., 1978). Together, these findings indicate that the way in which cries are categorized (pain or non-pain), the specific cause of the cry (i.e. hunger or frustration) or the method for elicitation are all factors that may influence the acoustic properties of subsequent cries. The subtle differences in methodology or lack of detail used to explain coding criteria for acoustic variables provides additional challenges when comparing observations across studies.

In the present study, there was also no significant difference between the two groups on researcher-rated Perceived Level of Distress for HR and LR infants. Venuti et al. (2012) provided evidence of enhanced brain activation in adults when processing recordings of cries of infants later diagnosed with ASD. Their research indicated that characteristics of cries from infants at-risk of ASD (i.e. higher F0 and shorter duration) may be more negatively experienced by caregivers and may be perceived as more distressing. While this observation was not supported, the current study was limited by using researcher ratings that may have been influenced by their familiarity with caregiving (Esposito et al., 2015). Future research may consider employing Magnetic Resonance Imaging as an alternative method for detecting subtle changes in perceptions of distress or negative experience.

HR and LR groups were significantly different in their performance on the MSEL and the ADEC assessments which were completed at the 12-month follow-up. HR infants obtained higher scores on the ADEC, which indicates that more ASD symptoms were endorsed. It is important to note that whilst the HR group have obtained elevated ADEC scores, relative to the LR group, a mean of 7.59 is still within the “low risk” range according to the ADEC scoring criteria. However, this observation suggests that at 12-months old, these HR infants, at a group level, are displaying more symptoms of ASD irrespective of whether they later receive a diagnosis. HR infants also obtained lower scores on the MSEL, which suggests that their developmental milestones are slightly behind their LR peers at 12-months old. Follow-up of these HR infants until diagnostic age will provide further insights on their cognitive behavioural development including ASD symptomatology.
Using a naturalistic method of data collection, we found that HR infants had shorter cry utterances, elevated ADEC scores and lower MSEL scores, relative to LR infants. The absence of any differentiation between groups on the remaining acoustic variables that were investigated appears inconsistent with previous findings for pitch variables in at-risk infants. Thus far, it appears that the method of elicitation, infants’ internal cues, and the categorizing of variables influences the subsequent acoustic analysis. Detailed reporting of the methods used in acoustic analyses is required in order to further this area of research.

To our knowledge, this was the largest prospective study to have collected acoustic cry data from infants at-risk of ASD. However, we acknowledge that the sample size under investigation is still relatively small, which limited the statistical power. However, six (24%) of HR infants were found to reach a clinical threshold for ASD on the ADOS-G at two years of age, which is consistent with previous estimates of risk for ASD among siblings of ASD probands. To maximise our statistical power, we examined 12-month behavioural variables in relation to continuous ADOS-G scores, though it is possible that a larger sample is still required to identify clear relationships over time. A key strength of the study was the closely matched HR and LR groups.

There were no significant differences in pregnancy variables such as smoking or alcohol use during pregnancy. Nor were there any differences in paternal or maternal education level, or in sex of the infants between groups. There was only a slight difference in maternal age at conception, with mothers conceiving at an older age in the HR group. Parents collected all cry recordings within the home which facilitated the naturalistic method. One limitation of this methodology was the minimal reporting of infant positioning during a cry episode. Due to the competing demands already placed on parents and caregivers, it was an additional task for them to record their infant crying and report details of their child’s position. Limited recording of these details, known to influence the quality of cry recordings, has been a weakness in this area of research and may best be addressed in the future by collecting samples of infant cries within a clinical setting. Due to the naturalistic method of data collection, it was not possible to control for the distance between the infant and the recording device when analysing measures such as the amplitude. However, all of the participants were given detailed recording instructions, including both verbal and written guidance, so the impact of this is likely to be minimal.
This study provides support for a unique acoustic profile in 12-month old infants at high-risk of ASD. Shorter cry duration has been replicated across multiple studies indicating that it may be a stable difference amongst infants at risk for disorder. Further research is required to better understand the physiology of shorter cries and also determine how these cries influence parental perception and response to their infant’s cry.
References


of the strange situation procedure in infant siblings at high risk for ASD. *Journal of Autism and Developmental Disorders*, 44(4), 975-980.


CHAPTER 6   General Discussion

The overarching objective of this thesis was to investigate potential behavioural and neurobiological risk markers for ASD in at-risk siblings during prenatal and early postnatal life. Chapter 1 reviewed the existing literature and highlighted two key areas that warranted further research. First, there were few studies of prenatal risk factors, despite growing evidence supporting neurological and growth differences in newborns later diagnosed with ASD (e.g. Gillberg & Souza, 2002; Hobbs et al., 2007). Second, although a broad range of potential behavioural markers of ASD have been identified during the first year of life (Dobkins, Akshoomoff, Carver, Dorhmann, & McCleery, 2008; Veness et al., 2011; Zwaigenbaum et al., 2005), few of these have been replicated, and prior to age one, no single risk factor is known to be predictive of a diagnosis of ASD. This thesis utilized an ASD sibling cohort design, which facilitated the investigation of early pre- and postnatal risk markers for ASD. The first aim of this thesis was to investigate the role of prenatal brain overgrowth in ASD. The second aim was to determine when the behavioural differences between high-risk (HR) and low-risk (LR) infant siblings first emerge, and the third aim was to study which of these behavioural characteristics, if any, predicted ASD outcomes at age two. In this chapter, the main findings addressing these three aims of this thesis are reviewed, followed by a discussion of the implications of the results, limitations of the reported studies and directions for future research (see also Table 20 for an overview of the findings presented in this thesis).
Table 20. Summary of key findings from each chapter

<table>
<thead>
<tr>
<th>Chapter</th>
<th>N</th>
<th>Purpose</th>
<th>Key findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two</td>
<td>n = 23 HR; 36 LR</td>
<td>To prospectively investigate fetal growth using two-dimensional ultrasound in a sample of fetuses at HR and LR of ASD</td>
<td>HR and LR infants did not differ in the rate of prenatal head and body growth throughout the second and third trimester of pregnancy.</td>
</tr>
<tr>
<td></td>
<td>missing n = 10 HR; 8 LR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three</td>
<td>n = 19 HR; 31 LR</td>
<td>This study utilized the three-dimensional ultrasound technique to report on a preliminary investigation of fetal brain growth between HR and LR fetuses.</td>
<td>Contrary to findings in Chapter two, fine-grained analyses of prenatal brain growth revealed an atypical pattern of cerebral growth across the second prenatal trimester in HR fetuses, relative to LR fetuses. Interestingly, the four individuals who displayed the ASD phenotype at age two years did not appear to drive this between-groups difference.</td>
</tr>
<tr>
<td></td>
<td>missing n = 14 HR; 13 LR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Four</td>
<td>n = 31 HR; 42 LR</td>
<td>A comparison of behavioural observations between 6-month-old HR and LR infants</td>
<td>At 6-months old, observations of HR infants were differentiated from those of LR infants on global ratings of negative affect. HR infant affect was rated as significantly more ‘negative’ compared to LR infant affect.</td>
</tr>
<tr>
<td></td>
<td>missing n = 2 HR; 2 LR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Five</td>
<td>n = 23 HR; 33 LR</td>
<td>To examine for differences in the acoustic properties of cries in 12-month-old HR and LR infants</td>
<td>At 12-months old, HR infants recorded significantly shorter cry durations, relative to LR infants. Furthermore, the six infant siblings who received a diagnosis of ASD at age two had amongst the shortest recorded cry durations.</td>
</tr>
<tr>
<td></td>
<td>missing n = 10 HR; 10 LR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The role of prenatal brain overgrowth in ASD

The first aim of this thesis was to shed further light on early prenatal brain development in HR and LR fetuses. To address this aim, Chapter 2 examined prenatal head and body size from prospective two-dimensional ultrasound scans of fetuses at HR (n = 23) and LR (n = 36) for ASD, as part of the PRISM study. Fetal ultrasound imaging was obtained at three time-points throughout pregnancy: 17-21 weeks, 22-25 weeks and 27-32 weeks gestation. Growth measurements were then compared for the HR and LR fetuses. We observed no statistically significant differences between the HR and LR fetuses on ultrasound measurements of head circumference (HC), biparietal distance (BPD), occipitofrontal distance (OFD) or femur length (FL), nor were there any significant differences in the rate of prenatal head and body growth throughout the second and third trimester. Analyses of head circumference (HC) uniformity and ratios of head growth relative to body size also revealed no differences between the HR and LR fetuses. Of note, especially given the small sample size, the mixed model beta values trended towards significance for the HR group to have slightly smaller growth measurements but faster growth rate relative to the LR group.

The null findings reported in Chapter 2 are broadly consistent with retrospective studies comparing routine ultrasound biometry between children later diagnosed with ASD and typically developing children (Hobbs et al., 2007; Whitehouse, Hickey, Stanley, Newnham, & Pennell, 2011). However, Whitehouse et al. (2011) did identify subtle disturbances in the uniformity of fetal brain growth in the ASD group, noting that several children with ASD had a relatively large head compared to body size, yet we observed no such difference in this sample. One hypothesis for the absence of this finding in our sample could relate to the use of an at-risk cohort, opposed to a sample of individuals with ASD, whereby any growth abnormalities may be more subtle or only present in amongst a sub-group within the sample of at-risk siblings.

Chapter 3 extended on the findings presented in Chapter 2 and provided a preliminary insight into a more detailed analysis of prenatal brain growth trajectory utilizing three-dimensional ultrasound technology. Using the same sample of participants (i.e. the PRISM study sample), we obtained three-dimensional ultrasound of cerebral volume for 19 HR fetuses and 31 LR fetuses at two time points during pregnancy (17-21 weeks, 22-25 weeks). Among HR fetuses we observed what appears
to be a unique growth pattern characterized by comparatively slow growth during gestational weeks 17-21, which is then followed by an accelerated period of growth up until gestational week 25. Whilst this is a preliminary finding in a small sample, this observation warrants further investigation. Due to the small sample size, we were unable to use this growth curve to predict ASD classification at follow-up. Thus we could only report graphical observations of the data. Participants that appeared to be driving the quadratic curve were not amongst the siblings who received a clinical diagnosis of ASD or met classification according to ADOS-G at 24-months old. These are purely observations of the data, and a larger sample size is required to statistically examine the relationship between prenatal growth and later ASD outcomes amongst at-risk siblings.

To our knowledge, this is the first study to report on prospective observations between HR and LR fetuses during the prenatal stages. These observations between the HR and LR fetuses are consistent with several studies reporting postnatal head circumference growth in children with ASD (Hazlett et al., 2017; Courchesne, Carper, & Akshoomoff, 2003; Gillberg & Souza, 2002). Not all studies in this area have consistently reported atypical brain growth trajectories in infants later diagnosed with ASD (e.g. Zwaigenbaum et al., 2014). The inconsistent results between studies suggests that head overgrowth may be a risk factor that is not common across all individuals who are later diagnosed with ASD but rather, a unique biomarker to a subgroup of individuals who meet criteria for this disorder. Furthermore, brain growth measurements may be indicative of neurodevelopmental characteristics that unique to this sub-group. We believe that the data presented in Chapter 3 are a unique contribution to the field and provide a first insight into prenatal brain development amongst HR siblings. The next step for this area of research relies upon large scale studies employing technologies such as magnetic resonance imaging to accurately pinpoint the onset of unique brain development in ASD and to further our understanding of the clinical utility of brain overgrowth as a marker for ASD (e.g. Hazlett et al., 2017).

The potential mechanisms that may underpin atypical prenatal neurodevelopment in ASD remain unclear. Courchesne and Pierce (2005) proposed a brain growth dysregulation hypothesis, whereby a period of postnatal atypical acceleration in growth is then followed by abnormally slow growth in the prefrontal cortex. Interestingly, this hypothesis is similar to the results we presented prenatally, however, a period of slow growth preceded a “catch up” period of accelerated growth.
Researchers have related the phenomena of atypical brain growth to the differences in neuroconnectivity observed amongst individuals with ASD (e.g. Courchesne & Pierce, 2005. Amongst some, but not all, brains of individuals with ASD there is thought to be an abundance of short-range connections and limited long-range connections (Courchesne & Pierce, 2005). Post-mortem studies have provided preliminary evidence in support of this theory, whereby brains of individuals with ASD appear to have an excess of minicolumns (Casanova, Buxhoeveden, Switala, and Roy, 2002) as well as discrete patches of disorganized matter in the prefrontal and temporal cortices (Stoner et al., 2014). The biological mechanism that underlies atypical neuroconnectivity and maturation of cerebral matter is unclear. Baron-Cohen et al. (2015) observed preliminary evidence of increased concentrations of sex steroids in amniotic fluid during pregnancy, as one such biological mechanism underlying neurodevelopmental abnormalities in ASD, whereby, exposure to high levels of testosterone in utero may indirectly restrict brain growth (Estrada, Varshney, & Ehrlich, 2006; Yang et al., 2002).

In conclusion, Chapter 3 reported on a novel ultrasound method to examine prenatal brain volume growth in fetuses at increased risk for ASD. This is the first study to shed some light on prospective prenatal neurodevelopment in a sibling cohort, and whilst there was some evidence indicative of a unique brain growth pattern in HR fetuses, these differences did not predict diagnosis at 24-months old. Due to the small sample size, these preliminary findings require replication in a larger, independent cohort before any conclusions can be drawn regarding prenatal brain growth in ASD. The observations of prenatal growth reported in this thesis highlight that this critical period of development warrants further investigation in the aetiological study of ASD.

When do behavioural differences between high- and low-risk infant siblings first emerge?

The second aim of this thesis was to determine when the behavioural differences between HR and LR infant siblings first emerge. The empirical data presented in Chapter 4 explored whether HR and LR infants were differentiated on behavioural measures obtained at 6- and 12-months old. The longitudinal study collected data from 31 HR and 42 LR infants. Data presented in Chapter 4 replicated a previous study by Wan et al. (2012) that identified differences in global MACI ratings between HR and LR infants at 6-months of age. From MACI ratings recorded from the videos of parent-
child interactions, we observed more frequent episodes of negative affect amongst HR infants, relative to LR infants, but this difference in observed affect was not identified in parent-report measures.

Furthermore, neither the parent-report nor detailed parent-infant interaction observations at 6-months were predictive of ASD outcome at two-years of age. By contrast, at 12-month follow-up, measures of developmental ability, social-communication and ASD-risk (MSEL, CSBS DP, ADOS-G, respectively) were all predictive of scores on the ADOS-G (the current gold standard ASD outcome assessment) at age two. These observations provide further replication that current formal and informal assessments of ASD-risk and developmental milestones at age one year, but not earlier, are predictive of later ASD symptomatology (Christensen et al., 2010; Ozonoff et al., 2010; Wan et al., 2013). Data from Chapter 4 also replicated a large body of empirical research that has identified differences between HR and LR infants at 12-months of age (Dobkins et al., 2008; Macari et al., 2012; Veness et al., 2011). Yet, this study reported no evidence that the behavioural measures (both parent-report and researcher-administered assessments) obtained at 6-months of age differentiated at-risk from low-risk siblings, nor were these measures predictive of ASD outcomes at age two. These observations provide further evidence that prior to 12-months of age, evidence supporting the clinical utility of ASD risk identification is weak, and ASD cannot currently be reliably indicated prior to 12-months of age in an at-risk cohort. Despite the limitations of small sample size and missing data in Chapter 4, this research is significant in that it highlights the potential for the parent-child paradigm to inform research of the subtle differences in social development during early infancy amongst siblings at-risk of ASD. The clinical utility of the parent-child paradigm prior to 12-months of age is yet to be determined, and poses an important question for future research.

Chapter 5 extended the findings for the 12-month period by comparing the acoustic properties of infant crying at 12-months old between HR and LR infant siblings. Acoustic cry recordings were collected by parents at the 12-month follow up for 23 HR and 33 LR infants. Cry duration was found to be significantly shorter for the HR group relative to the LR group, which is consistent with previous research (Esposito & Venuti, 2009; Esposito, Rostagno, Venuti, Haltigan, & Messinger, 2014), and further still, individuals with shortest cry durations obtained scores within the moderate-risk range for ASD on the ADEC and some, but not all of these individuals, met ASD
classification at 24-months old. These results provide some preliminary evidence for unique characteristics of infant cry amongst siblings of children with ASD. Again, the small sample size limits the generalizability of these findings, and prevented reliable statistical analyses of the relationship between cry duration and ASD outcomes. Interestingly, we observed no significant differences between the HR and LR infants in measures of fundamental frequency, first and second formants or in the amplitude of the cries. These observations deviate from the findings of previous research that has reported higher fundamental frequency measurements for HR infants, relative to LR infants (e.g. Esposito et al., 2014; Sheinkopf, Iverson, Rinaldi, & Lester, 2012). We hypothesized that that the way in which cries are categorized (pain or non-pain), the specific cause of the cry (i.e. hunger or frustration) and/or the method for elicitation, are all factors that may influence the acoustic properties of subsequent cries. Given that parents were responsible for obtaining cry recordings in the current study, the length of cry recordings and the number of cry recordings was variable. Thus, if we were to separate cries into “pain” and “non-pain” this would have further reduced the statistical power of the sample. A potential avenue for future research could involve providing families with small voice recorders (e.g. Lena™ technology) that could be kept in their infant’s clothes or close to their cot. Studies using this type of technology are showing promising results for identifying differences in language and communication between children with ASD and children who are language delayed (Dykstra et al., 2012; Oller et al., 2010).

**Do behavioural characteristics of high-risk infants at 6- and 12-months old predict ASD outcomes at age two?**

The third and final aim of this thesis was to evaluate whether any of the 6- or 12-month behavioural markers were predictive of a later diagnosis of ASD at age two. In the study reported in Chapter 4, at the two year postnatal follow-up, caregivers completed the CSBS DP and the HR and LR infant siblings were administered the MSEL and the current ‘gold standard’ of ASD outcome assessment, the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000) module one. Infant siblings were then classified according to one of three criteria; Autism, Autism Spectrum or none.
By age two, HR infants were differentiated from LR infants on a broad range of standardized and informal assessments of ASD risk. Data collected at 12-months old, but not at 6-months old, were found to predict ASD classification at age two. At two-year follow up, the HR group were differentiated from LR infants on a number of measures, including lower scores on the CSBS DP (total score and risk-category) and MSEL, and higher ADOS-G severity scores. Also, six HR infants compared to one LR infant met ADOS-G criteria for ASD. None of the measures collected at 6-month follow-up were significantly correlated with behavioural measures collected at two-year outcome. However, at 12-months, the MSEL, CSBS DP and ADEC scores were all significantly correlated with ADOS-G severity, CSBS DP and MSEL scores obtained at two years old.

Furthermore, for the study reported in Chapter 5, at two-year follow-up, the HR group were differentiated from LR infants on a number of measures, including lower scores on the MSEL (indicative of poorer performance), and higher ADOS-G severity scores (indicative of elevated ASD-risk). The six infants who met ADOS-G criteria for ASD at two years old recorded some of the shortest cry durations at 12-months old. There was a trend towards significance for shorter cry durations to be associated with more severe ADOS-G restricted and repetitive behaviours severity score and poorer performance on the MSEL language subscales. These results provide some preliminary evidence for a unique acoustic profile characterized by shorter overall duration of cries in infants at-risk of ASD.

Implications, limitations, future directions and final conclusions

The findings presented in this thesis have several clinical and research implications, particularly with regard to the early detection and intervention of infants at-risk of ASD. We observed preliminary evidence of differences between HR and LR siblings during the prenatal period from ultrasound scans of cerebral brain volume. The HR and LR group were differentiated on one behavioural observation obtained at 6-months of age. However, behavioural differences between the HR and LR groups were not predictive of ASD until 12-months of age. Future research is required to understand why there are these behavioural differences between the HR and LR groups independent of ASD outcome, and whether they are involved in the aetiological pathway to ASD.
These findings indicate that the symptoms of the broader autism phenotype are present long before ASD-risk is currently detected, which is largely consistent with previous research (Barbaro & Dissanayake, 2013; Bent, Dissanayake, & Barbaro, 2015; Dobkins et al., 2008; Landa, Holman, & Garrett-Mayer, 2007; Macari et al., 2012; Veness et al., 2011). A significant issue with delayed age of diagnosis (average age of 4.5 years old in Australia), is that by the time a child has been identified and diagnosed with ASD, many of the best opportunities for therapies to capitalise upon brain plasticity very early in development are not realised (Dawson, 2008). From the data presented in this thesis, it is apparent that differences between HR and LR siblings can be detected from as early as 12-months old, which is well before the average age of diagnosis in Australia. To overcome the large gap between symptom onset and diagnosis, Whitehouse (2017) proposed a new clinical pathway for ASD whereby children are identified as ‘at-risk’ of ASD by trained health professionals in the community, and are provided with evidenced-based early intervention. This clinical model has been suggested as an alternative to the current system in Australia, which dictates that a formal diagnosis of ASD is required, prior to commencement of intervention (Whitehouse, 2017).

Despite the presence of early signs of ASD prior to diagnosis, this thesis did not identify any behavioural risk factors that could reliably differentiate HR and LR infants prior to 12-months old that were predictive of later ASD diagnosis. An absence of behavioural markers prior to 12-months of age is a relatively consistent observation in the literature (Rozga et al., 2011; Young et al., 2009), where only a handful of small studies have reported that parent-report measures and behavioural observations obtained at 6-months-old were predictive of ASD outcomes (e.g. Jones & Klin, 2013; Sacrey et al 2015). One possible explanation for this relates to the many challenges associated with observing and quantifying infant behaviour. During testing sessions, infants cry, are easily fatigue, often require changing and feeding and regularly seek comfort from their caregiver. All of these developmentally appropriate behaviours pose challenges to standardizing infant assessment. Given the extensive research, and inconsistent observations of 0-12-month-old infant siblings to date, it could be argued that research efforts may be better spent on large replication studies examining potential behavioural markers that have shown the most promise in infants 12-months of age and older. Only then can we address the issue of poor specificity and sensitivity of behavioural observations, and improve the clinical utility of such measurements.
The sibling cohort design facilitates longitudinal assessment of potential risk markers. Consequently, due to difficulties recruiting participants for longitudinal studies, and that parents of children who receive an ASD diagnosis often choose not to have more children (Hoffmann et al., 2014; Wood et al., 2014), this design tends to rely on a small number of participants. Small sample size and missing data at follow-up largely limit the clinical utility of any potential marker that is assessed. Given the heterogeneous nature of HR sibling cohorts, with only a small percentage of infants receiving a diagnosis of ASD, it is unlikely that there will be an observable behaviour that is unique to this group (i.e. differentiated from LR infants), and is a reliable predictor of ASD diagnosis prior to age one. Analysing a sibling cohort relies on a ‘bottom up’ approach to the research, as it compares groups based on ‘high risk’ status. However, in a small sample of HR infants, few will receive a diagnosis of ASD, making it difficult to analyse risk markers that are predictive of later outcomes. Future research may consider using the ‘bottom up’ approach and identifying sub-groups of infants within the HR cohort based on factors that have been linked to increased risk of ASD such as early language delay, premature birth, low Apgar scores, or pregnancy complications.

Given the substantial increase in sibling cohort studies and ASD-risk, researchers have questioned the validity of extrapolating findings from these samples to infants at-risk of ASD in the general population. In a recent study, Sacrey et al. (2017) examined this research question by comparing clinical symptomatology between a group of infant siblings later diagnosed with ASD and a community sample of children identified with ASD (n = 86 in each group). Sacrey et al. (2017) observed several differences between the two groups including a greater number of females and less severe symptomatology amongst children in the sibling sample, relative to the community group. These important differences have implications for generalisability of findings from a sibling cohort. Throughout prospective sibling studies, infants are assessed regularly and comprehensively. Often the differences in symptomatology that are observed between groups are subtle in nature and identified by trained ASD-specific clinicians and researchers. Contrastingly, children identified at-risk in the community experience a different referral process whereby child health nurses, caregivers or extended family members have noticed early signs of atypical development or delayed milestones. Such differences between these groups may render findings from sibling studies as unique to this cohort and limit generalisability in identifying risk markers for
ASD. To our knowledge, Sacrey et al. (2017) have produced the first study to examine these differences between subgroups of at-risk children, whereby outcome measures included the ADOS-G (Lord et al., 2000), Autism Diagnostic Interview-Revised (Lord et al. 1994) and Vineland Adaptive Behaviour Scales (Sparrow et al. 1984, 2005). Further research investigating the clinical characteristics of these groups may wish to consider employing some examination of neurodevelopment using tools such as magnetic resonance imaging or eye tracking to identify whether observations from sibling cohorts are generalizable to a community sample.

Furthermore, clinical utility continues to be an issue, where few of the behavioural markers that are examined in this literature are transferable to a clinical setting. For example, these variables (e.g., frequency of eye-contact) often cannot be interpreted independently as indicators of ASD, and need to be organised into reliable screening tools (e.g. Sacrey et al., 2016). One potential avenue to increase clinical utility of the research to date, and to support families prior to their infant’s first birthday, could involve providing parents with information that details evidence-based risk factors to date, and informs parents of what signs to look out for, and when to seek a referral for further assessment of ASD risk. Should research continue to focus on identifying subtle nuances in infant behaviour between groups (e.g. frequency of duration of eye-contact and positive/negative affect), it is unlikely that clinical utility, as well as sensitivity and specificity of these risk markers will improve. Early risk marker research appears to be moving its focus towards improving clinical utility by making use of more objective tools and advanced technology, such as eye-tracking devices. Use of Magnetic Resonance Imaging to identify neurodevelopmental atypicalities in early life is one such example (e.g. Hazlett et al., 2017).

Another limitation of risk marker research and the current thesis, is that analyses rarely differentiate between early signs that are uniquely related to ASD from signs of other developmental disorders or delay. Several studies have reported that compared to children with developmental delay, children with ASD use less frequent requesting, responding to name, social smiling, following of pointing, functional play and joint attention (Trillingsgaard et al., 2005). Further, Watson et al. (2013) observed at-risk infants’ gesture use and reported that infants later diagnosed with ASD were less likely to use gesture to initiate joint attention or to regulate behaviour, relative to infants with developmental delay or typical development. Despite observations of small differences in early social markers between children with ASD and children with developmental
delay, other studies have reported that while some social markers recorded prior to 12-months of age are predictive of ASD as compared with language impairment and/or typical development, these social communication domains did not predict ASD diagnosis when compared with developmental delay up until 24 months of age (Veness et al., 2014). Further research is required to identify which markers reflect general vulnerability for developmental delay and which markers are specific to ASD. One such method involves recruiting infants who have been identified at-risk by a paediatrician or child health nurse following routine screening for communication delays or via parental self-referral. These cohorts may include a larger proportion of at-risk infants who receive a diagnosis of ASD or developmental/language delay at follow-up, which is required to investigate potential risk markers that accurately differentiate developmental delay from ASD.

**Final conclusions**

From the data presented in this thesis, it is apparent that differences between the HR and LR siblings can be detected reliably from as early as 12-months old. Parent-child interaction paradigms are a promising methodological approach for identifying subtle disruptions in early social communication development in at-risk cohorts. Whilst differences at 6-months old were not predictive of ASD at two years old, the reported group difference indicates that early disruptions in interaction style, such as elevated negative affect, may be more characteristic of the broader autism phenotype. Understanding how these characteristics may be involved in the aetiological pathway for ASD is a key area for future research. From 12-months old, HR and LR infants are differentiated on several informal and formal assessments of ASD-risk. Performance on formal behavioural measures was also predictive of later ASD outcomes. This is consistent with a large body of research that has identified risk markers of ASD well before a diagnosis is received. Given that only a few siblings of children with ASD will actually receive a diagnosis of ASD (approx. 20%; Ozonoff et al., 2011), larger studies of community risk samples are required to determine which behavioural markers are reliable indicators of risk.

This thesis also presented the first preliminary evidence of prenatal differences in cerebral volume between HR and LR fetuses. While the sample size is small, these data indicate that atypical cortical size and growth trajectory during the second trimester
is not present in all individuals who are diagnosed with ASD. Nevertheless, we believe that the significant difference in growth trajectory between the HR and LR groups provides an interesting observation for the field. Given that siblings of ASD probands, irrespective of phenotype, may display biological differences that can inform our understanding of the etiological pathways to ASD (Ahmed & Vander Wyk, 2013; Belmonte et al., 2010; Spencer et al., 2012), this finding requires further investigation.
References


Green, J., Charman, T., Pickles, A., Wan, M. W., Elsabbagh, M., Slonims, V., … Jones,


Appendix I

A 'bottom-up' approach to aetiological research in autism spectrum disorders

Autism Spectrum Disorders (ASD) are currently diagnosed in the presence of impairments in social interaction and communication, and a restricted range of activities and interests. However, there is considerable variability in the behaviours of different individuals with an ASD diagnosis. The heterogeneity spans the entire range of IQ and language abilities, as well as other behavioural, communicative and social functions. While any psychiatric condition is likely to incorporate a degree of heterogeneity, the variability in the nature and severity of behaviours observed in ASD is thought to exceed that of other disorders. The current paper aims to provide a model for future research into ASD subgroups. In doing so, we examined whether two proposed risk factors – low birth weight (LBW), and in-utero exposure to selective serotonin reuptake inhibitors (SSRIs) – are associated with greater behavioural homogeneity. Using data from the Western Australian Autism Biological Registry, this study found that LBW and maternal SSRI use during pregnancy were associated with greater sleep disturbances and a greater number of gastrointestinal complaints in children with ASD, respectively. The findings from this ‘proof of principle’ paper provide support for this 'bottom-up' approach as a feasible method for creating homogenous groups.

Autism Spectrum Disorders (ASD) are currently diagnosed in the presence of impairments in social interaction and communication, and a restricted range of activities and interests. However, there is considerable variability in the behaviours of different individuals with an ASD diagnosis. Traditionally, researchers have conceptualised ASD as a unitary disorder with a large spectrum, and have sought to discover a single aetiological factor that leads to disorder. However, the behavioural heterogeneity has been mirrored at the genetic level, for instance, many susceptibility loci have been identified, yet each has been found to account for a small amount of variance only (1-2%) (Weiss et al., 2008). A proposition that has gathered momentum over the last decade involves moving away from the traditional conceptualisation of ASD as a unitary disorder towards conceptualising a syndrome of multiple and separate disorders;
in essence, re-examining ‘autism’ as ‘the autisms’ (Geschwind and Levitt, 2007; Whitehouse and Stanley, 2013).

Research in this area has traditionally adopted a ‘top-down approach’ by constraining behavioural phenotypes in the hope that this will facilitate the identification of biological subtypes. For example, Buxbaum et al. (2001) reported linkage evidence for a susceptibility gene for Autistic Disorder on chromosome 2. In an analysis of 95 affected-relative pair families with Autistic Disorder they found a maximum multipoint heterogeneity LOD score (HLOD) of 1.96 and a maximum multipoint NPL score of 2.39 on chromosome 2q (at 186cM, for D2s364). When families were grouped according to delayed onset (at age >36 months) of phrase speech, linkage to chromosome 2 increased (HLOD = 2.99, NPL = 3.32). Shao et al. (2002) found further evidence for a susceptibility gene on chromosome 2. In an analysis of 82 sibling pairs with Autistic Disorder they found a HLOD of 0.53 at D2S116. When the analysis was restricted to a subset of 45 families with phrase speech delay (>36 months), linkage to chromosome 2q increased (HLOD = 2.12). Whilst this approach has received the most attention in aetiological research, generally speaking, it has underperformed, with only weak evidence that stratification based on IQ, age at first word, or verbal ability yield a more genetically homogenous population (Geschwind and Levitt, 2007).

A ‘bottom-up’ approach to identify biological subtypes of ASD has not received the same level of research attention. This methodology focuses on known aetiological risk factors, and whether individuals exposed to these risk factors have a more homogenous phenotype. In this paper, we report on this bottom-up approach, focusing on aspects of the phenotype that are not part of the core defining features of the disorder. We know that comorbid medical conditions are highly prevalent in ASD (Bauman, 2010). Sleep problems are thought to affect 40-80% of children on the spectrum (Richdale, 1999) and estimates of gastrointestinal disorders in ASD range from 9-70% (Buie et al., 2010). The high prevalence of these comorbid conditions in children with ASD may suggest the presence of important genetic and/or biological markers, which if identified, can refine our ability to be more precise in categorising clinical and genetic subtypes within the autism spectrum (Bauman, 2010). In this paper, we have adopted a bottom-up approach by stratifying groups based on two previously identified risk factors, namely, maternal use of selective serotonin reuptake inhibitors (SSRIs) during pregnancy and low birth weight (LBW). The second part of our strategy
involved examining the homogeneity within the groups based on medical complaints such as sleep problems and gastrointestinal complaints in addition to core features of ASD such as social behaviour, language characteristics and severity.

SSRI use during pregnancy has gained considerable attention over the last two years and is thought to be implicated in an increased risk of ASD diagnosis (Croen et al., 2011). Prevalence studies in the US estimate that up to 8% of mothers may be treated with SSRIs during pregnancy for conditions such as anxiety disorders or major depression (Alwan et al., 2011). SSRIs act primarily by blocking the serotonin transporter, thereby raising extracellular serotonin (5-HT) levels (Oberlander et al., 2009). These SSRIs readily cross the placental and blood-brain barriers to the fetus, with the potential to alter central 5-HT signalling (Oberlander et al., 2009). The neuroactive properties of SSRIs are thought to be a potential risk to fetal neurodevelopment, since 5-HT plays such a critical role in regulating diverse processes such as cell division, differentiation, migration, myelination, synaptogenesis and dendritic pruning (Gaspar et al., 2003). A number of researchers have hypothesised that the increase in ASD diagnoses in recent years may be associated with a commensurate increase in maternal use of anti-depressant medication during pregnancy (Croen et al., 2011). A recent population-based case-control study by Croen et al. (2011) reviewed record-based data describing the postnatal development of children exposed to SSRIs in utero. This study examined data for children born through a medical care program during the period of 1995-1999. Infants in the sample who were later diagnosed with ASD were considered cases. Children without an ASD diagnosis were randomly sampled from the remaining cohort at a ratio of 5 control children per 1 case child. Using this matched sample, Croen et al. (2011) investigated SSRI use throughout pregnancy and found that 70 women who took anti-depressant medication the year before the birth of their child had twice the risk of having a child with ASD (n = 20 offspring with ASD, 28.57%) compared with 1735 women who did not take any anti-depressant medication (n = 278 offspring with ASD, 16.02%).

Using a similar population-based nested case-control design, Rai et al. (2013) investigated the extent to which parental depression and maternal antidepressant use during pregnancy were associated with ASDs in offspring. For parental depression, record-based data was available for 4429 cases of ASD and 43277 age- and sex-matched controls, and for maternal antidepressant use, data existed for 1679 ASD cases and 16845 non-ASD controls. They found that a history of maternal but not paternal
depression was associated with higher risk of autism in offspring. These associations were largely limited to children of mothers who reported using antidepressants at the first antenatal interview. Antidepressant use during pregnancy was reported by 1.3% of mothers of children with ASD and by 0.6% of control mothers, equating to an almost twofold increase in risk of ASD with use of antidepressants (Rai et al., 2013). Other studies that have examined the effect of maternal SSRI use during pregnancy have observed several atypical behavioural outcomes among offspring, including delay in meeting gross motor milestones (Pedersen et al., 2010), a wide range of feeding difficulties (Oberlander et al., 2006) and sleep disturbances (Zeskind and Stephens, 2004).

Low birth weight (<2500g) has also been considered an environmental risk factor implicated in a range of psychiatric disorders including ASD, anxiety disorder and depression (Indredavik et al., 2004; Jaspers et al., 2012; Gardener et al., 2011). Lampi et al. (2012) examined data from the case-control Finnish Prenatal Study of Autism and ASDs and found that children with very low birth weight (<1500g) had a greater than three-fold increased odds of autism compared with children with normal birth weight (2500-3999g). Interestingly, LBW did not significantly increase the odds of Asperger syndrome (Lampi et al., 2012). In addition to these associated psychiatric disorders, when compared to children with normal birth weight (NBW), children with LBW have been found to show pervasive motor impairments, increased socio-emotional issues, increased risk of sleep-disordered breathing and reductions in language ability (de Kieviet et al., 2009; Scott et al., 2012; Spittle et al., 2009; Paavonen et al., 2007; Barre et al., 2011). Despite evidence supporting the role of these environmental risk factors in the development of ASD, no single factor has been identified that poses a determinant risk for this disorder.

This paper will adopt a ‘bottom-up’ approach to parsing ASD heterogeneity by investigating the behavioural phenotype associated with two possible environmental risk factors. The first study compared the behavioural and developmental phenotype of children with ASD whose mothers used SSRIs during pregnancy with the phenotype for a tightly matched group of children with ASD whose mothers did not use SSRIs during pregnancy. It was hypothesised that those children with ASD whose mothers used SSRIs during pregnancy would display early feeding and sleep disturbances compared to the control group of children with ASD. We also examined whether these children showed a distinguishable behavioural phenotype. Study 2 compared the phenotype of
children with ASD born with LBW with a matched group of children with ASD born with NBW. It was hypothesised that those LBW children with ASD would display greater sleep disturbances (e.g. sleep-disordered breathing), language difficulties and socio-emotional problems compared to the NBW group. This ‘proof of principle’ study seeks to examine two potential risk factors within the context of a ‘bottom up’ research design. If the hypotheses are supported this paper may provide a blueprint for using the 'bottom-up' approach as a feasible method for creating homogenous groups compared with the more costly 'top-down' approach which requires large sample sizes.

Study 1
Materials and Methods

Participants

Participants were part of the Western Australian Autism Biological Registry (WAABR), which is an ongoing study of children with a clinical diagnosis of an ASD and their families taking place at the Telethon Institute for Child Health Research in Perth, Western Australia (see Taylor, Maybery & Whitehouse, in press). Diagnosing ASD in Western Australia mandates assessment by a clinical team comprising a Pediatrician, Psychologist and Speech-Language Pathologist under DSM-IV guidelines (American Psychiatric Association, 1994). A diagnosis is only made when there is consensus amongst the team. The current study included nine participants from the WAABR whose mothers reported SSRI use during pregnancy (cases). Each of these participants was individually matched on gender and chronological age at assessment (within 15 months) with three further children with ASD (n = 27) whose mothers did not take an SSRI during pregnancy.

Measures and Procedure

Prior to attending a face-to-face assessment, families were mailed and asked to complete a comprehensive case-history questionnaire relating to the mother’s pregnancy and the ASD child’s development. Mothers were asked to provide details of any history of psychological disorder such as major depression or anxiety. They were also asked to provide the name of any prescription or non-prescription medications, the dosage, and the amount they used during pregnancy. A series of questionnaires were also included
in this package, including the Social Responsiveness Scale (SRS; Constantino and Gruber, 2002), Children’s Sleep Habits Questionnaire (CSHQ; Owens et al., 2000), Children’s Communication Checklist-2 (CCC-2; Bishop, 2003) and a gastrointestinal complaints questionnaire (Ibrahim et al., 2009).

The SRS is a 65-item questionnaire used to examine a range of social behaviours characteristic of ASD in children over the last 6 months. A total score can be calculated for the SRS as well as five subscale scores, namely, social communication, autism mannerisms, social motivation, social awareness and social cognition. Parents respond using a four-point scale ranging from “not true” (1) to “almost always true” (4). A higher total score on this measure is indicative of greater social difficulties. The CSHQ is a 34-item parent-report instrument that was used to examine sleep behaviour over a ‘typical week’. Parents were asked to rate how often their child showed behaviours such as “struggle at bedtime” and “show fear at sleeping alone” using a 1-3 point scale corresponding to “rarely”, “sometimes” or “usually”, respectively. A total score and eight subscale scores (bedtime resistance, sleep onset delay, sleep duration, sleep anxiety, night wakings, parasomnias, sleep disordered-breathing and daytime sleepiness) can be calculated for responses on the CSHQ. Higher total scores on the CSHQ indicate that the child has a greater number of sleep problems.

The CCC-2 is a parent-report questionnaire designed to assess the communication skills of children aged 4-16 years. The purposes of the CCC-2 are the identification of pragmatic language impairment, screening of receptive and expressive language skills, and assistance in screening for ASD. The CCC-2 consists of 70 items that are divided into 10 scales, each with 7 items. The first 4 scales focus on specific aspects of language and communications skills (content and form). The next 4 scales assess the pragmatic aspects of communication. The last 2 scales measure behaviours that are usually impaired in children with ASDs. The parent rates the frequency of the communication behaviour described in each item from 0 (less than once a week or never) to 3 (several times a day or always). Interpretation is based on a General Communication Composite (GCC), with lower scores indicative of greater language and communication difficulties.

Parents also completed a brief questionnaire related to their child’s history of gastrointestinal problems. This questionnaire was developed specifically for the WAABR case history questionnaire based on the list of complaints in Ibrahim et al.
After reviewing the literature related to gastrointestinal symptoms they identified five categories that have been reported to be common in patients with autism, namely, constipation, diarrhoea, gastro-oesophageal reflux or vomiting, abdominal discomfort/irritability or feeding issues (Ibrahim et al., 2009). If the parent reported their child had experienced any of the five gastrointestinal complaints for a period of at least a month, resulting in consultation with their doctor, they received a score of one for the indicated complaint(s). Any other reports received a score of zero. Using this scoring method these complaints were analysed in two ways: (1) individually to see if the frequency of each complaint differed between the two groups and (2) as a summary measure of gastrointestinal complaints (score of one or more) versus no gastrointestinal complaints (score of zero).

Families were then invited to the Telethon Institute for Child Health Research for a face-to-face behavioural assessment. Clinical diagnoses of ASD were confirmed using the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000). The present study used the child’s age and ADOS module (reflective of their quantity of speech) to calibrate severity scores (0-10) for each participant according to the severity scale of Gotham et al. (2009). This enabled comparisons between the participants, irrespective of the module they completed.

**Statistical analyses.**

Between-group differences in the quantitative scores of the SRS, CCC-2, CSHQ and ADOS severity scale were investigated with independent-samples t-tests. Responses to the gastrointestinal complaints questionnaire were analysed according to whether parents reported zero complaints or one or more complaints for their children using chi-square analyses with Fisher’s exact test of significance.

**Results**

The SSRI case (n = 9) and control (n = 27) groups did not significantly differ on gestational age ($F(1, 34) = 1.05, p > .05$) or maternal age at conception ($F(1, 34) = 3.45, p > .05$). Table 21 provides details of the maternal, pregnancy and offspring characteristics of the case group.
### Table 21. Study 1: Maternal and offspring characteristics of the SSRI case group

<table>
<thead>
<tr>
<th>SSRI taken during pregnancy</th>
<th>Maternal</th>
<th>Offspring</th>
<th>Gestational age at birth</th>
<th>Age at assessment</th>
<th>ADOS module administered</th>
<th>ADOS severity score</th>
<th>CSHQ score</th>
<th>SRS score</th>
<th>Number of gut problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1 Lexapro Daily</td>
<td>Major depression</td>
<td>41 weeks</td>
<td>5,6</td>
<td>2</td>
<td>1</td>
<td>42</td>
<td>146</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Case 2 Lexapro Daily</td>
<td>Major depression</td>
<td>40 weeks</td>
<td>4,6</td>
<td>2</td>
<td>6</td>
<td>62</td>
<td>157</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Case 3 Lovan 3 months</td>
<td>Major depression</td>
<td>36 weeks</td>
<td>5,2</td>
<td>2</td>
<td>8</td>
<td>54</td>
<td>158</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Case 4 Efexor Daily</td>
<td>Major depression</td>
<td>38 weeks</td>
<td>10,2</td>
<td>3</td>
<td>3</td>
<td>46</td>
<td>172</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Case 5 Not specified -</td>
<td>Major depression</td>
<td>38 weeks</td>
<td>4,3</td>
<td>2</td>
<td>7</td>
<td>59</td>
<td>166</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Case 6 Escitalopram Daily</td>
<td>-</td>
<td>38 weeks</td>
<td>2,9</td>
<td>1</td>
<td>4</td>
<td>77</td>
<td>207</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Case 7 Fluoxetine Daily</td>
<td>Anxiety disorder</td>
<td>40 weeks</td>
<td>8,5</td>
<td>3</td>
<td>3</td>
<td>63</td>
<td>145</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Case 8 Aropax 1 month</td>
<td>Anxiety disorder</td>
<td>39 weeks</td>
<td>3,1</td>
<td>1</td>
<td>6</td>
<td>54</td>
<td>120</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Case 9 Zoloft Daily</td>
<td>Major Depression</td>
<td>38 weeks</td>
<td>3,5</td>
<td>1</td>
<td>6</td>
<td>41</td>
<td>90</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Independent-samples t-tests revealed that there were no significant differences between the two groups on any of the SRS, CCC-2, CSHQ, or ADOS severity scores (Table 22). However, analysis of responses to the gastrointestinal complaints questionnaire found that mothers who used SSRIs during pregnancy were more likely to have a child with ASD who experienced one or more gut problems (n = 8, 88.9%), compared to the control group (n = 13, 48.1%), $\chi^2(1), p = .05$. To further investigate this association, chi-square analyses with Fisher’s exact test were performed on the five individual complaints (Table 23). The individual complaints did not significantly differentiate between the groups. However, the percentage of constipation complaints was noticeably larger (though, not significantly) for cases compared to controls.

Table 22. Study 1: Descriptive statistics and independent-samples t-tests for CSHQ, SRS and ADOS severity scores.

<table>
<thead>
<tr>
<th></th>
<th>SSRI Cases</th>
<th>Controls</th>
<th>Statistic</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M$</td>
<td>SD</td>
<td>$M$</td>
<td>SD</td>
</tr>
<tr>
<td>CSHQ</td>
<td>55.56</td>
<td>11.36</td>
<td>51.58</td>
<td>11.96</td>
</tr>
<tr>
<td>Bedtime resistance</td>
<td>9.56</td>
<td>2.79</td>
<td>9.83</td>
<td>3.25</td>
</tr>
<tr>
<td>Sleep onset delay</td>
<td>1.89</td>
<td>.60</td>
<td>1.83</td>
<td>.87</td>
</tr>
<tr>
<td>Sleep duration</td>
<td>5.11</td>
<td>2.42</td>
<td>4.96</td>
<td>2.39</td>
</tr>
<tr>
<td>Sleep anxiety</td>
<td>4.89</td>
<td>1.45</td>
<td>4.79</td>
<td>1.82</td>
</tr>
<tr>
<td>Night wakings</td>
<td>6.00</td>
<td>1.50</td>
<td>5.04</td>
<td>2.03</td>
</tr>
<tr>
<td>Parasomnias</td>
<td>11.33</td>
<td>2.78</td>
<td>9.58</td>
<td>3.36</td>
</tr>
<tr>
<td>Sleep dis-breathing</td>
<td>4.11</td>
<td>1.83</td>
<td>4.08</td>
<td>1.56</td>
</tr>
<tr>
<td>Daytime sleepiness</td>
<td>12.67</td>
<td>3.16</td>
<td>11.46</td>
<td>2.95</td>
</tr>
<tr>
<td>SRS</td>
<td>151.22</td>
<td>32.84</td>
<td>145.11</td>
<td>25.86</td>
</tr>
<tr>
<td>Social awareness</td>
<td>16.56</td>
<td>1.81</td>
<td>16.44</td>
<td>2.94</td>
</tr>
<tr>
<td>Social cognition</td>
<td>28.78</td>
<td>8.23</td>
<td>26.56</td>
<td>6.24</td>
</tr>
<tr>
<td>Social communication</td>
<td>50.33</td>
<td>12.58</td>
<td>47.74</td>
<td>8.88</td>
</tr>
<tr>
<td>Social motivation</td>
<td>25.56</td>
<td>5.36</td>
<td>24.93</td>
<td>5.95</td>
</tr>
<tr>
<td>Autistic mannerisms</td>
<td>30.00</td>
<td>7.78</td>
<td>29.44</td>
<td>7.54</td>
</tr>
<tr>
<td>ADOS severity</td>
<td>4.89</td>
<td>2.26</td>
<td>5.93</td>
<td>1.96</td>
</tr>
<tr>
<td>CCC-2</td>
<td>30.75</td>
<td>6.99</td>
<td>36.15</td>
<td>16.53</td>
</tr>
</tbody>
</table>

\[ t(31) = .86  .40 \]

\[ t(34) = .57  .57 \]

\[ t(34) = 1.32  .19 \]

\[ t(15) = .63  .54 \]
Table 23. Study 1: Chi-square analyses using Fisher’s exact test for both groups of children for the five gastrointestinal complaints.

<table>
<thead>
<tr>
<th>Gut complaint</th>
<th>SSRI Cases</th>
<th>Control</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>Constipation</td>
<td>4(44.4)</td>
<td>4(14.8)</td>
<td>.09</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>2(22.2)</td>
<td>3(11.1)</td>
<td>.58</td>
</tr>
<tr>
<td>Gastro reflux</td>
<td>2(22.2)</td>
<td>3(11.1)</td>
<td>.58</td>
</tr>
<tr>
<td>Abdominal</td>
<td>1(11.1)</td>
<td>2(7.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>Feeding</td>
<td>5(55.6)</td>
<td>8(29.6)</td>
<td>.24</td>
</tr>
<tr>
<td>One or more complaints</td>
<td>8(88.9)</td>
<td>13(48.1)</td>
<td>.05</td>
</tr>
</tbody>
</table>

**Discussion**

This is the first study to examine the relationship between SSRI exposure and ASD phenotype. There were no differences between the cases and individually matched control participants in scores on the SRS, CCC-2, CSHQ or ADOS-G severity. However, children with ASD whose mothers took SSRIs during pregnancy were significantly more likely to experience gastrointestinal complaints during childhood. Further examination of the relationship between gastrointestinal complaints and in utero SSRI exposure revealed that no individual complaint could significantly differentiate the two groups. While this does not support Oberlander et al. (2009) who found evidence for feeding disturbances in typically developing infants exposed to SSRIs in utero, it is possible that the small sample size contributed to the null findings for the less-frequent individual complaints.

The current study was limited by the absence of a control group of children whose mothers had affective disorders but who did not take SSRIs during pregnancy, and therefore we are unable to parse out whether the differences in the frequency of gut problems is related to mood disturbances or SSRI use. Rai et al. (2013) reported an association between maternal depression and an increased risk of offspring ASD. Although they found that this association was largely confined to antidepressant use in a subsample of mothers, future studies could build on the findings presented here and in Rai et al. (2013) by comparing the phenotype for children with ASD whose mothers report untreated depression during pregnancy with a matched ASD control group of
children. The hypothesised association between ASD and gastrointestinal pathology is the subject of increasing amounts of research. Despite the numerous parental reports of gastrointestinal complaints among their children with ASD, studies have failed to find a significant difference in the prevalence of these complaints between children with ASD and control groups of children (e.g. Ibrahim et al., 2009). The current findings suggest that SSRI exposure in utero may be one potential candidate accounting for variance in the gut phenotype in children diagnosed with ASD.

**Study 2**

**Materials and Methods**

**Participants**

The study involved using data for 16 participants from WAABR whose birth weight was ≤ 2500g (LBW). Each of these participants was individually matched on gender and chronological age at assessment (within 18 months) with two further control children with ASD (n = 32) whose birth weight was within the normal range (NBW;2500g-3999g).

**Measures and Procedure**

Within the case-history questionnaire, mothers were asked to report their child's birth weight. For the purposes of Study 2, data collected for each child using the SRS, CSHQ, ADOS severity, CCC-2 and gastrointestinal complaints questionnaire were analysed.

**Statistical analyses**

Between-group differences in the quantitative scores of the SRS, the CSHQ, CCC-2 and ADOS severity scale were investigated with independent-samples t-tests.

Responses to the gastrointestinal complaints questionnaire were analysed using chi-square analyses with Fisher’s exact test of significance.
Results

The LBW (n = 16) and the NBW (n = 32) groups did not significantly differ on maternal age at conception ($F(1, 45) = .07, p > .05$). Mean gestational age was significantly lower for the LBW group ($F(1, 43) = 28.53, p < .05, M = 34.25$ weeks, SD = 4.55 weeks) relative to the NBW group ($M = 39.07$ weeks, SD = 1.33 weeks, $p < .05$).

Table 24 provides details of the offspring characteristics of the case group. Independent-samples t-tests (see Table 25) revealed that LBW children with ASD had significantly higher scores relative to the NBW group on the CSHQ for Total Sleep Disturbance and two of the subscales, namely, Sleep-Disordered Breathing and Daytime Sleepiness. There were no significant differences between the two groups on the SRS, CCC-2 or ADOS severity scores (Table 25).
### Table 24. Study 2: Offspring characteristics of the LBW case group

<table>
<thead>
<tr>
<th>Case</th>
<th>Birth weight</th>
<th>Gestational age at birth</th>
<th>Age at assessment</th>
<th>ADOS module</th>
<th>ADOS severity score</th>
<th>CSHQ score</th>
<th>SRS score</th>
<th>GCC</th>
<th>Number of gut problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>600</td>
<td>24</td>
<td>11;1</td>
<td>3</td>
<td>4</td>
<td>45</td>
<td>152</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>895</td>
<td>27</td>
<td>7;4</td>
<td>2</td>
<td>6</td>
<td>57</td>
<td>166</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>985</td>
<td>29</td>
<td>5;6</td>
<td>1</td>
<td>3</td>
<td>39</td>
<td>133</td>
<td>38</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1565</td>
<td>30</td>
<td>4;7</td>
<td>1</td>
<td>6</td>
<td>42</td>
<td>172</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1640</td>
<td>37</td>
<td>5;2</td>
<td>1</td>
<td>6</td>
<td>65</td>
<td>169</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>1665</td>
<td>37</td>
<td>5;2</td>
<td>2</td>
<td>4</td>
<td>56</td>
<td>143</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1725</td>
<td>35</td>
<td>14;4</td>
<td>3</td>
<td>4</td>
<td>63</td>
<td>171</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>1765</td>
<td>34</td>
<td>2;8</td>
<td>1</td>
<td>7</td>
<td>47</td>
<td>107</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>2097</td>
<td>40</td>
<td>13;1</td>
<td>1</td>
<td>6</td>
<td>54</td>
<td>183</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>2285</td>
<td>36</td>
<td>5;2</td>
<td>2</td>
<td>8</td>
<td>54</td>
<td>158</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>2300</td>
<td>37</td>
<td>5;11</td>
<td>1</td>
<td>7</td>
<td>51</td>
<td>163</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>2426</td>
<td>37</td>
<td>9;7</td>
<td>1</td>
<td>8</td>
<td>69</td>
<td>200</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>2450</td>
<td>32</td>
<td>4;7</td>
<td>2</td>
<td>6</td>
<td>52</td>
<td>159</td>
<td>56</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>2500</td>
<td>38</td>
<td>4;3</td>
<td>2</td>
<td>7</td>
<td>59</td>
<td>166</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>2125</td>
<td>37</td>
<td>11;3</td>
<td>3</td>
<td>6</td>
<td>66</td>
<td>191</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>2480</td>
<td>38</td>
<td>4;6</td>
<td>2</td>
<td>5</td>
<td>66</td>
<td>156</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 25. Study 2: Descriptive statistics and independent-samples t-tests for CSHQ, SRS, CCC-2 and ADOS severity score.

<table>
<thead>
<tr>
<th></th>
<th>LBW</th>
<th>NBW</th>
<th>Statistic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>M</td>
<td>SD</td>
</tr>
<tr>
<td>CSHQ</td>
<td>55.31</td>
<td>9.08</td>
<td>47.84</td>
<td>8.84</td>
</tr>
<tr>
<td>Bedtime resistance</td>
<td>9.81</td>
<td>2.76</td>
<td>8.13</td>
<td>2.38</td>
</tr>
<tr>
<td>Sleep onset delay</td>
<td>1.88</td>
<td>.72</td>
<td>1.52</td>
<td>.68</td>
</tr>
<tr>
<td>Sleep duration</td>
<td>5.69</td>
<td>2.39</td>
<td>4.55</td>
<td>1.93</td>
</tr>
<tr>
<td>Sleep anxiety</td>
<td>4.63</td>
<td>1.63</td>
<td>4.74</td>
<td>1.86</td>
</tr>
<tr>
<td>Night wakings</td>
<td>5.19</td>
<td>1.87</td>
<td>4.19</td>
<td>1.66</td>
</tr>
<tr>
<td>Parasomnias</td>
<td>10.44</td>
<td>2.71</td>
<td>9.97</td>
<td>2.56</td>
</tr>
<tr>
<td>Sleep dis-breathing</td>
<td>4.31</td>
<td>1.49</td>
<td>3.29</td>
<td>.82</td>
</tr>
<tr>
<td>Daytime sleepiness</td>
<td>13.38</td>
<td>3.36</td>
<td>11.19</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social awareness</td>
<td>17.31</td>
<td>2.73</td>
<td>17.97</td>
<td>2.33</td>
</tr>
<tr>
<td>Social cognition</td>
<td>30.25</td>
<td>3.64</td>
<td>28.77</td>
<td>5.81</td>
</tr>
<tr>
<td>Social communication</td>
<td>54.00</td>
<td>8.25</td>
<td>51.13</td>
<td>8.58</td>
</tr>
<tr>
<td>Social motivation</td>
<td>27.69</td>
<td>4.70</td>
<td>26.68</td>
<td>4.88</td>
</tr>
<tr>
<td>Autistic mannerisms</td>
<td>32.56</td>
<td>7.16</td>
<td>31.68</td>
<td>7.36</td>
</tr>
<tr>
<td>ADOS severity</td>
<td>5.81</td>
<td>1.47</td>
<td>6.56</td>
<td>1.98</td>
</tr>
<tr>
<td>CCC-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCC</td>
<td>36.78</td>
<td>13.92</td>
<td>28.88</td>
<td>13.67</td>
</tr>
</tbody>
</table>

Similarly, children with LBW (n = 16, 81%) did not experience significantly greater gastrointestinal issues compared to the NBW group (n = 32, 53%), \(\chi^2(1), p = .07\). To further investigate this association, chi-square analyses with Fisher’s exact test were performed on the five individual complaints (Table 26. Study 2:Chi-square analyses using Fisher’s exact test for LBW (n = 16) and NBW (n = 32) groups of children for the five gastrointestinal complaints.). The individual complaints did not significantly differentiate between the groups.
Table 26. *Chi-square analyses using Fisher’s exact test for LBW (n = 16) and NBW (n = 32) groups of children for the five gastrointestinal complaints.*

<table>
<thead>
<tr>
<th>Gut complaint</th>
<th>LBW N (%)</th>
<th>NBW N (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constipation</td>
<td>7(43.8)</td>
<td>9(28.1)</td>
<td>.22</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>2(12.5)</td>
<td>3(9.4)</td>
<td>.55</td>
</tr>
<tr>
<td>Gastro reflux</td>
<td>5(31.3)</td>
<td>8(25)</td>
<td>.45</td>
</tr>
<tr>
<td>Abdominal</td>
<td>0(0)</td>
<td>4(12.5)</td>
<td>.19</td>
</tr>
<tr>
<td>Feeding</td>
<td>9(56.3)</td>
<td>13(40.6)</td>
<td>.24</td>
</tr>
<tr>
<td>One or more complaints</td>
<td>8(50)</td>
<td>13(40.6)</td>
<td>.05</td>
</tr>
</tbody>
</table>

**Discussion**

The second study examined the phenotype of children with ASD born with LBW relative to a group of children with ASD born with NBW. This study did not observe any significant differences between the groups on the SRS, ADOS-G severity, or CCC-2. This is inconsistent with findings of greater socio-emotional issues and reduced language ability in LBW children compared to NBW children in the absence of an ASD diagnosis (Scott et al., 2012; Barre et al., 2011). There was a trend towards significance for the gastrointestinal complaints questionnaire, whereby the LBW group reported more frequent complaints compared with the NBW group. The present study did find that children in the LBW group obtained higher mean scores on the CSHQ for total sleep disturbance, Daytime Sleepiness and Sleep-Disordered Breathing relative to the NBW group. This supports the finding of sleep-disordered breathing in children with LBW without an ASD diagnosis (e.g. Paavonen et al., 2007). Interestingly, compared to norms from typically developing children ($M = 3.24$) and children with ASD ($M = 3.92$) (Hoffman et al., 2006), LBW children with ASD obtained larger mean scores for the Sleep-disordered Breathing subscale ($M = 4.31$).

Currently, there are no norms to describe performance of typically developing LBW children on the CSHQ. It would be interesting to compare sleep disturbance between LBW typically developing children and LBW children with ASD. Thus it may be useful to conduct a more comprehensive study of LBW and NBW children with and without ASD to look more closely at the significance of the present findings.
Unsurprisingly, the LBW children had a significantly lower gestational age at birth than the NBW children, which raises the possibility that gestational age may be driving the findings and not birth weight. However, it is important to note that the study by Lampi et al. (2012), which informed our hypotheses, found that low birth weight was a better predictor of ASD diagnosis than was prematurity.

**General discussion**

This present study used a ‘bottom-up’ approach to seek understanding of the heterogeneity of ASD by investigating the behavioural phenotype associated with two suspected environmental risk factors, namely, in-utero SSRI exposure and LBW. It was hypothesised that children with ASD who were exposed to one of these environmental risk factors would present with a more homogenous phenotype relative to individually-matched control groups of children with ASD. There was some preliminary support for this hypothesis. While the children in the LBW and SSRI exposed groups were no different to their respective control groups in quantitative and qualitative measures of the core symptomatology of autism, there was evidence that the two groups were distinct in the level of their non-core symptomatology such as sleep and gastrointestinal complaints, respectively.

The numbers of children with ASD in the ‘aetiological risk’ subgroups are small, and therefore we urge caution in drawing conclusions from these data. Rather, we seek to highlight a different method for understanding the heterogeneity in the ASD phenotype. We believe that the preliminary findings of increased levels of non-core symptoms of ASD among certain ‘aetiological risk’ subgroups, provides evidence that this ‘bottom-up’ methodology may assist ASD research. Studies including larger samples of children with ASD will build on the research presented here, and provide the opportunity to validate our preliminary findings.

Whilst the present study did not find any differences in core ASD symptoms between LBW and SSRI-exposed children with their respective control groups, we know that each child who is given an ASD diagnosis presents with the triad of core symptoms irrespective of their severity. It is unlikely that a single environmental factor could be attributed to ‘causing’ one of these core impairments. Rather we may expect that the interplay between the environment and a child’s genetic profile contributes to
the variable expression of autistic-related traits (Ratajczak, 2011). Therefore, it seems reasonable that environmental factors may be related to the expression of non-core ASD symptoms among these children rather than to any variance in core symptomatology.

Recently, Whitehouse and Stanley (in press) reaffirmed an emerging view in the literature with regard to reconceptualising autism in moving away from a unitary disorder with one cause, and towards an ‘umbrella’ for a collection of behavioural disorders resulting from a range of causal pathways. In their paper they describe how research in cerebral palsy may be analogous to research on autism. Initially cerebral palsy was thought to be a unitary disorder caused by anoxia secondary to trauma occurring during labour and delivery. However, the heterogeneity in symptoms and severity amongst children with cerebral palsy led researchers to hypothesise that there may be many causal pathways. Many other causes were identified for cerebral palsy following this reconceptualisation, such as complications of preterm birth, infections and inflammation in utero (McIntyre et al., 2012). For diagnosis, cerebral palsy is now considered an umbrella term covering a wide range of syndromes that arise secondary to a number of brain lesions/anomalies occurring early in development (Badawi et al., 2008).

A key question facing the field is whether the long-held view that autism is a unitary disorder with a single causal pathway is correct, or whether autism may best be conceptualised as an umbrella term for a collection of behavioural disorders resulting from a range of causal pathways, analogous to cerebral palsy. Current evidence suggests that the latter may be a more accurate representation. Heterogeneity in the distal causes of autism is now well-established. It is estimated that between 10 and 15% of individuals with autism have a known genetic aetiology, but the loci and nature of these lesions vary, from known syndromes to observable cytogenetic lesions and rare de novo mutations (e.g., copy number variations) (Abrahams and Geschwind, 2009). Among those with idiopathic autism, no single genetic risk variant has been found to occur in more than 1% of individuals (Abrahams and Geschwind, 2009). Similarly, environmental risk factors identified through epidemiological studies and examined in this study - in utero exposure to selective serotonin reuptake inhibitors (Croen et al., 2011) and low birth weight (Lampi et al., 2012) – differ considerably in the hypothesized biological paths to disorder, and as yet, no known environmental exposure is deterministic of autism.
Given that diagnosis is currently based on behaviour, the question of whether autism is one or multiple disorders is ultimately a query over the proximal causes of these behaviours, and one perhaps best addressed in neuroscience. Neuroscientific studies may help determine whether (a) distal risk factors ‘fan in’ on a common neurobiological substrate that has the capability of underpinning the considerable behavioural heterogeneity in autism (one disorder), or (b) the exact combination of distal risk factors determines the brain regions and functions that are affected, which in turn prescribe the behavioural profile of each individual (multiple disorders). A key research aim will be to investigate the correspondence (if any) between known distal (genetic and environmental) and proximal (neurobiological) risk factors for autistic behaviours, using increasingly sophisticated environmental monitoring, genetic sequencing, and neuroimaging techniques.

Using preliminary data in this study we have demonstrated how a ‘bottom-up’ approach can be applied to current aetiological research. Grouping individuals using this method may facilitate the identification of subtypes of people with ASD. Elucidating the underlying nature of the disorder(s) is a crucial step towards achieving perhaps the ‘holy grail’ of autism research: tailoring intervention to the biological and cognitive makeup of each individual (Whitehouse and Stanley, in press).
References


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