THE DIFFERENTIAL IMPACT OF OXYTOCIN RECEPTOR (OXTR) GENOTYPES ON THE RISK OF AUTISM SPECTRUM DISORDER (ASD) AND RESULTING SOCIAL COMMUNICATION DEFICITS

by

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Abstract

**Background:** Autism spectrum disorder (ASD) is multifactorial and is likely the result of complex interactions between multiple environmental and genetic factors. Recently, it has been suggested that each symptom cluster of the disorder, such as poor social communication, may be mediated by different genetic influences. Genes in the oxytocin pathway, which mediates social behaviours in humans, have been studied with single nucleotide polymorphisms (SNPs) in the oxytocin receptor gene (OXTR) being implicated in ASD. This thesis examines the presence of different oxytocin receptor genotypes, and their associations with ASD and resulting social communication deficits.

**Methods:** The relationship between four OXTR variants and ASD was evaluated in 607 ASD simplex (SPX) families. Cases were compared to their unaffected siblings using a conditional logistic approach. Odds ratios and associated 95 percent confidence intervals were obtained. A second sample of 235 individuals with a diagnosis of ASD was examined to evaluate whether these four OXTR variants were associated with social communication scores on the *Autism Diagnostic Interview – Revised* (ADI-R). Parameter estimates and associated 95 percent confidence intervals were generated using a linear regression approach. Multiple testing issues were addressed using false discovery rate adjustments.

**Results:** The rs53576 AG genotype was significantly associated with a lower risk of ASD (OR = 0.707, 95% CI: 0.512-0.975). A single genotype (AG) provided by the rs2254298 marker was found to be significantly associated with higher social communication scores (Parameter estimate = 1.833, SE = 0.762, p = 0.0171). This association was also seen in a Caucasian only and mothers as the respondent samples. No association was significant following false discovery rate adjustments.

**Conclusion:** The findings from these studies provide limited support for the role of OXTR SNPs in ASD, especially in social communication skills. The clinical significance of these associations remains unknown, however, it is likely that these associations do not play a role in the severity of symptoms associated with ASD. Rather, they may be important in the appearance of social deficits due to the rs2254298 markers association with enlarged amygdalas.
Co-Authorship

This thesis is the work of Meagan Milton in collaboration with her supervisors, Drs. Hélène Ouellette-Kuntz and Xudong Liu. Meagan Milton developed the study design with assistance and input from Drs. Hélène Ouellette-Kuntz and Xudong Liu. Dr. Xudong Liu currently runs the Autism Spectrum Disorders – Canadian-American Research Consortium. Participant identification was done by Meagan Milton, with the aid of Melissa Hudson, and genotyping was carried out by Dr. Amy McNaughton. Data analyses were conducted by Meagan Milton, under the supervision of Drs. Hélène Ouellette-Kuntz and Xudong Liu. This thesis, and the manuscripts contained within, was written by Meagan Milton with feedback from Drs. Hélène Ouellette-Kuntz and Xudong Liu.
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<th>Full Form</th>
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<tbody>
<tr>
<td>ADHD</td>
<td>Attention Deficit Hyperactivity Disorder</td>
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<td>ADI-R</td>
<td>Autism Diagnostic Interview-Revised</td>
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<tr>
<td>ADOS</td>
<td>Autism Diagnostic Observation Schedule</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism Spectrum Disorder</td>
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<tr>
<td>ASD-CARC</td>
<td>Autism Spectrum Disorders – Canadian-American Research Consortium</td>
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<tr>
<td>CDD</td>
<td>Childhood Disintegrative Disorder</td>
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<tr>
<td>CNV</td>
<td>Copy Number Variants</td>
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<tr>
<td>Cu²⁺</td>
<td>Copper</td>
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<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>DSM-III</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Third Edition</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition</td>
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<tr>
<td>DSM-IV-TR</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision</td>
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<tr>
<td>DSM-5</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy-Weinberg Equilibrium</td>
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<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
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<tr>
<td>ICD-10</td>
<td>International Classification of Diseases, Tenth Edition</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage Disequilibrium</td>
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<tr>
<td>MPX</td>
<td>Multiplex</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>OXTR</td>
<td>Oxytocin Receptor Gene</td>
</tr>
<tr>
<td>PDD-NOS</td>
<td>Pervasive Developmental Disorder – Not Otherwise Specified</td>
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<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>SPX</td>
<td>Simplex</td>
</tr>
<tr>
<td>ToM</td>
<td>Theory of Mind</td>
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<tr>
<td>Zn²⁺</td>
<td>Zinc</td>
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Chapter 1

Introduction

1.1 Background and Rationale

Autism spectrum disorder (ASD) is an umbrella term used to describe a group of neurodevelopmental disorders of empathy [1]. There are two main symptom domains that characterize ASD: (1) social interaction and communication, and (2) restrictive behaviour [1]. Currently, the three conditions to which ASD refers to are: autism, Asperger’s disorder, and pervasive developmental disorder-not otherwise specified. They are one of the most common disorders in children, with approximately one in 100 Canadian children meeting the criteria for at least one of the conditions [1]. Despite its prevalence, the underlying aetiology of this disabling condition remains largely unknown. Researchers have demonstrated that ASD is multifactorial and is likely the result of complex interactions between environmental and genetic factors [2,3]. Bailey et al. (1995) found that 60 percent of monozygotic twins were concordant for ASD compared to zero percent of dizygotic twins [2]. In 2011, Hallmayer et al. replicated these earlier results, finding a concordance rate of 58 percent among male monozygotic twins compared to 21 percent for male dizygotic twins [3]. Fifty-eight percent of the variance in this sample could also be explained by shared environmental factors among the twins [3]. While there is evidence of a strong genetic link in the aetiology of ASD, it remains unclear as to where in the genetic code the issue is arising. Researchers have demonstrated that specific symptom clusters may be the result of mutations (polymorphisms) of genes within different neuronal pathways [4].

This thesis examines the relationship between polymorphisms in the oxytocin receptor gene (OXTR) and social communication deficits. Among individuals with ASD, social communication is characterized by deficits in verbal and non-verbal behaviours used in reciprocal social interaction [5].

Impairments in the oxytocin neuropeptide system, specifically within the OXTR, have been found to contribute to the ‘asociality’ that is typically exhibited in individuals with ASD. However, these studies have been exploratory [4-9]. More specifically, plasma oxytocin levels have been found to be lower in individuals with ASD indicating lower social scores in these individuals [10]. Therefore, it is hypothesized that polymorphisms in the OXTR may affect oxytocin’s ability to bind to its receptor and exert its downstream effects. If this hypothesis is true, the level of oxytocin may be lower leading to the occurrence of social communication deficits in individuals with ASD. Studies evaluating this relationship have been done with small samples in specific ethnicities, and have found conflicting evidence regarding the presence of this relationship and its strength [6,7,8,12,13]. As such, further genetic research is needed to evaluate the relationship between OXTR variants and social communication deficits. This thesis will further examine this potential relationship between ASD and OXTR.

1.2 Empirical Objectives

The purpose of this thesis is to contribute to existing knowledge of the aetiology of ASD and specifically the role of SNPs rs2254298, rs7632287, rs1042778 and rs53576, in the OXTR within the oxytocin system, through two empirical objectives:

1. To compare the genotype distributions of four OXTR variants in individuals with ASD to those in their unaffected siblings, and

2. To determine the association between specific OXTR genotypes and social communication deficits among individuals with a diagnosis of ASD.

1.3 Overview of Study Design

This study used data from the Autism Spectrum Disorders – Canadian-American Research Consortium (ASD-CARC) Research Registry [11]. The first objective used a case-control study design with a total of
1214 participants being evaluated (607 cases and 607 controls). Two hundred and seventy-five participants were used in the second objective that used a cross-sectional approach.

1.4 Thesis Organization

This thesis is presented in manuscript format. The second chapter, entitled literature review, provides an overview of current epidemiologic literature on ASD, its aetiology, and the relationship between the oxytocin system and ASD. It begins by describing the apparent increase in ASD prevalence, the history of ASD, changes made to the diagnostic criteria in the current Diagnostic and Statistical Manual of Mental Disorders (DSM-5), and how to screen for and diagnose ASD. Environmental factors, genetic factors, and potential gene-environment interactions that may contribute to the underlying aetiology of ASD are then described. Chapter two concludes with a discussion of the oxytocin neuropeptide and its receptor, the relationship between oxytocin and psychopathy, the neurobiology of ASD and oxytocin, and the relationship between OXTR, ASD and social communication deficits. The third chapter describes the study design and analytic approach of this thesis. The fourth chapter is a manuscript on the relationship between OXTR polymorphisms and the risk for ASD. The fifth chapter is a manuscript that describes the relationship between OXTR polymorphisms and social communication deficits in verbal individuals with ASD. Chapter six includes additional results that were not presented in the second manuscript. The final chapter provides a discussion of the general findings of this thesis, the strengths and limitations of the study, and the implications for future research. Three appendices contain additional materials referred to in the text.
1.5 References


Chapter 2

Literature Review

2.1 Epidemiology of Autism Spectrum Disorder

In the 1990s, early research estimated the prevalence of ASD as being less than 10 in 10,000 individuals [1,2]. Since then, the prevalence of the disorder has reportedly increased to approximately 100 in every 10,000 Canadian children [3]. However, researchers continue to debate whether or not this is the result of an actual increase in ASD resulting from increased risk or an artificial increase due to a number of other factors, specifically inaccurate diagnosis, different research methodologies, cultural factors, and a better understanding and diagnosis of the disorder [1,2,4-7].

Inaccurate diagnosis of ASD can lead to a higher reported prevalence of ASD than what is actually occurring in the population. For example, Barbaresi et al. (2009) concluded that inaccurate diagnosis of ASD accounted for an 8.2-fold [95% CI: 3.9 – 19.0] increase in research-derived diagnoses [8]. Of those research-identified cases, approximately 53 percent would not receive a clinical diagnosis of ASD under the Diagnostic and Statistical Manual of Mental Disorders (DSM), Fourth Edition (1994) [8]. Barbaresi et al. (2009) further elaborated on why inaccurate diagnosis of ASD is occurring. They point to the fact that numerous clinicians, including clinical psychologists, school psychologists, psychiatrists, and neurologists, are currently allowed to diagnose a child with ASD [1,8]. They suggest that this results in inaccurate diagnoses as the amount of experience and training each clinician has with individuals with ASD varies widely [1,8]. While in agreement with Barbaresi et al. (2009), Leonard et al. (2010) stated that the overlapping symptoms between ASD and other conditions (i.e. communication disorders) result in inaccurate diagnoses as well [9]. In addition, Leonard et al. (2010) explain that individuals will currently be diagnosed with ASD in the circumstances where symptoms overlap compared to the past.
where a diagnosis of an intellectual disability was more commonly given [9]. Thus, reported prevalence estimates of ASD may be artificially increased due to the inclusion of less accurate diagnoses [1].

The increase may also be the result of other factors besides inaccurate diagnosis. First, prevalence estimates may be affected by the use of different research methodologies across studies. For example, studies may use data from registries, retrospective accounts, telephone interviews, and/or whole-area surveys [1]. Each of the methods can result in different prevalence estimates that may not reflect the true prevalence of these disorders. Second, acculturation and related social factors may be responsible for the apparent increase in ASD prevalence over time. Acculturation refers to the merging of cultures as a result of prolonged contact. For example, a study by Kramer et al. (2004) examined the effect of acculturation and the prevalence of ASD in developed nations, as these rates have increased significantly over the last decade [1]. They found higher rates of ASD among Israeli born children compared to Ethiopian born children [10]. The researchers then examined Israeli children of Ethiopian extraction (immigrants to Israel) and found that these individuals had higher rates of ASD compared to Ethiopian born children [10]. This would contribute to an artificial increase in ASD as these cases always existed but remained undiagnosed due to social factors within the country.

Finally, the changing diagnostic criteria and assessment methodology are also believed to be responsible for much of the artificial increase in the prevalence of ASD [1]. While the characterization of ASD through different iterations of the DSM will be covered in the section below, each new version of the manual has included changes in the diagnostic criteria leading to different criteria being used in different studies potentially resulting in changes in prevalence estimates. The primary change that has resulted in an increase in prevalence estimates is the broadening of the diagnostic criteria [1]. In addition, the DSM is predominantly used in North America, whereas the *International Classification of Diseases* (ICD) is a classification system used worldwide. The current version, ICD-10, contains completely
different diagnostic criteria for ASD resulting in differential diagnoses between continents and different prevalence estimates across studies [1]. For example, a study in California, reported that approximately 26.4 percent [95% CI: 16.25 – 36.48] of the increase in ASD prevalence between 1992 and 2005 was attributable to changes in diagnostic criteria and assessment [11]. Additionally, in 2008, Coo et al. found that approximately one-third of the increase, in a study population in British Colombia, was the result of the movement of an individual from a category outside of ASD to ASD (also referred to as diagnostic substitution) [7]. Given these factors, it is evident that the increase in prevalence estimates could, in part, be attributed to these diagnostic practice factors.

Even after accounting for the above factors, research has indicated that the prevalence of ASD is increasing [12]. The true prevalence of ASD may be increasing due to two factors. Researchers suggest that improvements in diagnostic measures and an increase in general awareness of the disorder are responsible for a true increase in prevalence and allow for a more accurate estimate of the prevalence of ASD to be made [1].

2.2 History of Autism Spectrum Disorder

In 1943, Leo Kanner first described autistic disorder as a deviation from typical neurologic development in which children displayed an extreme preference for solitude, a need for sameness, persistent interests, repetitive behaviours, lack of imagination, and language difficulties [13,14]. In 1944, Hans Asperger described a condition similar to autistic disorder where the individual exhibited poor social and emotional functioning but seemed to be intellectually capable [13,14]. This condition became known as Asperger’s disorder. By DSM-III (1980), a diagnosis of autistic disorder required an onset of symptoms prior to 30 months and severe deficits in social relationships [13]. In the DSM-IV (1994), an individual must have impaired social interaction, either restricted or unusual behaviours, and cannot meet the criteria for autistic disorder to be diagnosed with Asperger’s [13,14]. Finally, in 2000 individuals who exhibit
characteristics similar to the above two conditions, but do not meet the criteria for diagnosis of either of them, were first classified as having pervasive developmental disorder – not otherwise specified (PDD-NOS) in the DSM-IV-TR [13,14].

By DSM-IV, the symptoms underlying ASD were referred to as a ‘triad of impairments’ [15]. The basic triad includes impairments in social interaction, social communication, and restricted and repetitive behaviour patterns [15]. Individuals who show a marked reduction in non-verbal signs of interest in being with another person, such as reduced eye contact, are said to have impairments in social interaction [15]. Impairment in social communication refers to a decrease in the ability of an individual to ‘converse’ non-verbally and verbally, share ideas and interests, and/or negotiate in a positive way with another person [15]. High frequency behaviours with invariant repetition and the need for the environment to remain the same comprise a group of behaviours referred to as restricted and repetitive behaviour patterns [16]. Ultimately, impaired social instinct is considered to be the fundamental issue in all of the ASD conditions. While these impairments are present at around 18 months of age, a diagnosis of ASD is rarely made before the age of two [17].

Two issues with the DSM-IV-TR emerged. First, since the main criteria and sub-criteria for each separate ASD condition were described in general terms, by the ‘triad of impairments’, it was very difficult to differentiate between the subtypes [15]. Thus, the validity of diagnosis, by both the DSM-IV-TR and the ICD-10, is assumed to be inadequate due to the large number of potential diagnoses [18]. Second, the criteria for symptom onset prior to age three meant that many cases of ASD were being missed when symptoms were not being observed until later in life [15,18]. For example, in some cases, the child has yet to even begin to show signs of ASD symptoms by the age of three and would not be diagnosed with ASD as a result [18]. The most recent version of the DSM, DSM-5 (2013), was released to ameliorate these issues.
2.2.1 DSM-5 Modifications

DSM-5 places ASD subtypes on a spectrum based on different levels of symptom severity rather than listing them as discrete conditions [15,18]. Thus, the DSM-5 uses the single diagnosis of ASD to refer to any of these three conditions: autism, PDD-NOS, and Asperger’s disorder [18]. ASD is now characterized by the severity of impairments in two main domains: (1) social interaction and communication, and (2) restrictive behaviour [19-22]. These domains have been specifically described for different age groups and more extensively characterize an individual’s clinical presentation [18]. While these terms still refer to the domains and impairments described above, the diagnostic criteria have changed considerably.

DSM-5 also removed the need for the onset of symptoms to occur before age three [18]. The deletion of this criterion has allowed adolescents and adults, presenting with these symptoms, to be diagnosed with an ASD [18]. This is because the recognition of symptoms may only occur when the requirement for social abilities increases, which occurs as an individual gets older [18]. However, it is still important that a diagnosis of ASD occur early in life for early intervention and a better outcome [18].

2.3 Screening and Diagnosis

To be diagnosed with an ASD, a child must ideally go through a series of tests occurring in two steps. However, it is important to note that while the following section outlines what is recommended, other tools can be used to give a diagnosis of ASD by a single clinician. First, it is recommended that developmental screenings, to test if children are meeting developmental milestones, occur when the child is nine, 18, and 24 and/or 30 months. These screenings have the child interact with the parent to evaluate how he/she learns, speaks, behaves, and moves. Specific screenings for ASD should occur at 18 and 24 months. If there is cause for concern at any of these screenings, the child is to be referred to the second step consisting of formal testing known as the comprehensive diagnostic evaluation [23,24].
A multidisciplinary team performs a comprehensive clinical assessment to gain information surrounding pregnancy, birth and developmental history. The Autism Diagnostic Interview-Revised (ADI-R) is a diagnostic tool that can be used to interview the parent (refer to Chapter 3 for an elaboration). The clinician should also ask if and when the parents noticed developmental regression in the child being evaluated. The child then undergoes a formal assessment, using the Autism Diagnostic Observation Schedule (ADOS), as well as an informal assessment, in natural settings.

Second, following the developmental assessment, as described in the preceding paragraph, the child will undergo a clinical evaluation that includes physical, neurologic and skin examinations. If the neurologic examination produces abnormal findings, further neuroimaging tests may be needed to exclude the possibility of other syndromes. Finally, laboratory tests are used to rule out Fragile X syndrome and other chromosomal anomalies prior to a diagnosis of ASD being made. After these steps are completed, and other potential comorbid disorders considered, a diagnosis of ASD is made generally by the time the child is three years of age [14,24,25]. It is also important to note that ASD can be diagnosed in the presence of other comorbid disorders, such as Fragile X.

There are many disorders that are comorbid with ASD, including seizure disorders, psychiatric disorders (i.e. attention deficit hyperactivity disorder [ADHD]), gastrointestinal disorders, intellectual disability, and auditory disorders and infections [26,27]. It is important to identify these comorbidities for two reasons. First, if they are identified and managed early enough, many of them are treatable and will result in an increased quality of life [27]. Secondly, and most notably, phenotypic and genetic clusters of individuals with ASD (forming different subtypes) may be found as a result of identifying medical disorders that involve a specific organ system [27]. As a result, causative and biological mechanisms can be better understood due to a more defined and meaningful subtype classification [27].
2.4 Aetiology of Autism Spectrum Disorders

Interest in the underlying aetiology of ASD has increased since Kanner first described autism in 1943. A key pathological feature of the autistic brain is the abnormal brain connectivity [28]. This connectivity is expressed through three components: (1) neural assemblies and long-range connectivity between brain regions, (2) physical connectivity between synapses and tracts, and (3) functional connectivity seen in neurotransmission [28]. With respect to ASD, researchers have shown that symptom expression is the product of high local connectivity with low long-range connectivity that has resulted from alterations in synaptic elimination, and/or the formation or changes in the inhibitory/excitatory synaptic ratios [28]. For example, abnormal activity of immune signaling that interferes with proper neuronal circuitry establishment during development contributes to the emergence of autistic phenotypes [28].

Importantly, these aberrations in brain connectivity could be the result of multiple different factors. Over the past 20 years, two different research foci, environmental and genetic, have been taken to identify potential factors relevant to this abnormal brain connectivity pathogenesis and subsequent development of ASD [28-30]. Recent research, using monozygotic and dizygotic twins, has indicated that environmental factors account for approximately 58 percent [95% CI: 30 – 80] of the risk factors associated with the development of ASD, whereas 38 percent [95% CI: 14 – 67] of the occurrence is the result of genetic factors [31]. However, previous studies have found evidence for a strong genetic link [29,31-33,47-50]. In 1995, Bailey et al. stated that the estimated heritability of autism was approximately 90 percent [31]. Another study conducted by Rutter (2002) indicated that genetic factors account for approximately 70 percent of the variance (different phenotypes) in ASD [29,32]. A third experimental focus, gene-environment interactions, has recently been suggested. It evaluates how both environmental and genetic factors combine with one another to produce ASD. This focus highlights that not enough is
known about either the environmental or genetic factors separately and thus, more research is needed in all foci to gain a better understanding of the underlying aetiology of ASD.

2.4.1 Environmental Factors

Environmental factors believed to be risk factors that prompt the development of ASD include heavy metal exposure, impaired immune functioning, and teratogens [30,33-36]. It has been demonstrated that proper brain functioning is reliant on metal ion homeostasis, and that severe neurological symptoms and cognitive diseases, such as ASD, result when this homeostasis is disrupted [34]. For example, copper ($\text{Cu}^{2+}$) levels have been shown to be significantly elevated in the nails and hair of individuals with ASD [34]. An overload of $\text{Cu}^{2+}$ within the body results in zinc ($\text{Zn}^{2+}$) deficiency due to their competing roles [34]. According to Faber et al. (2009), serum samples from individuals with ASD have demonstrated an increased $\text{Cu}^{2+}/\text{Zn}^{2+}$ ratio [34,37]. Yasuda et al. (2011) found that 43.7 percent of males and 52.5 percent of females with ASD, from a sample of 1,967 children three years of age or younger, were $\text{Zn}^{2+}$ deficient [34,38]. Based on these results, infantile $\text{Zn}^{2+}$ deficiency is considered to contribute to the pathogenesis of ASD [34,38].

Immune functioning can interfere with brain connectivity and produce symptoms associated with ASD [28,34]. Abnormalities in postnatal immune functioning have been attributed to prenatal exposure to either maternal stress hormones and/or psychological stress of the mother, and prenatal viral infection [39,40]. For example, Patterson et al. (2009) showed that \textit{in utero} viral or bacterial infections were linked to higher risks of ASD due to immunological imbalance and that these immunological changes persist throughout development and into adulthood in these individuals [34,41]. The increased risk of ASD that is associated with prenatal infection and perinatal stress is mediated by immune functions such as the proliferation of lymphocytes, natural killer cell activity, and cytokine production [34,41]. In addition,
complications during labour and delivery lead to cerebellar hemorrhagic injuries that are associated with higher rates of ASD [42].

Lastly, environmental toxins (teratogens) have the ability to cause birth defects and have been suggested as potential risk factors for ASD [34]. For example, prenatal exposure to valproic acid [43], which is an anticonvulsant agent, and thalidomide [44], which is an immunomodulatory agent, have been associated with higher incidence of ASD [34]. The in utero exposure to valproic acid results in developmental delays and deficits in social interaction due to its internal inhibition of histone deacetylase and reduced neuroligin-3 messenger RNA expression in the hippocampus [45]. It has also been shown that maternal use of psychiatric drugs, such as those prescribed for stress, is associated with the development of ASD indicating that these psychiatric drugs are another type of potential teratogen [46]. Regardless of the toxin, or the environmental factor, the concordance rate remains less than 100 percent indicating that a genetic predisposition must be at play as well.

2.4.2 Genetic Factors

Genetic factors are thought to largely account for the occurrence of ASD [34]. Twin studies, by Freitag (2006) and Ronald and Hoekstra (2011), reported a 60 to 90 percent estimate of heritability, which indicates a strong genetic link in the development of ASD [33,47-49]. Furthermore, two to three percent of families overall are multiplex (MPX; have more than one child with a diagnosed ASD) [29]. Recurrence rates of ASD in families that already have a single child with ASD is approximately four percent if the child is male and seven percent if the child is female [29]. This rate increases to between 33 and 50 percent for MPX families [29]. Research has identified different approaches to identifying the three different types of genetic factors that account for the occurrence of ASD, which are: rare chromosomal abnormalities, rare and de novo copy number variations, and common genetic variants [50].
Approximately five percent of ASD cases display visible chromosomal reorganization [50]. Duplication within the 15q11-q13 region of the Prader-Willi/Angelman syndrome region is the most common chromosomal abnormality associated with ASD [50]. It appears in one to three percent of ASD cases compared to the expected 0.1 to 0.5 percent [50]. Trisomy 21, 45 X Turner syndrome, 47 XYY, and 47 XXY are also associated with ASD to a lesser degree [50]. While it has been seen that chromosomal abnormalities are potential genetic factors, they are extremely rare and are unlikely to be the sole factor responsible. Thus, research has moved away from identifying them [50].

Rare or de novo copy number variations (CNVs) are genomic alterations that lead to either an abnormal amount of copies of one or more sections of the DNA acting as sequence-level mutations or resulting in a genomic disorder respectively. Both large and small changes associated with ASD have been found using CNVs screening [50]. This screening has demonstrated that around five to 10 percent of ASD cases show CNVs. For example, CNV-16p11.2, PTCHD1/PTCHD1AS, and NRXN1 have been found to be significantly associated with ASD [51]. Specifically, approximately one percent of ASD cases expressed either a microdeletion or microduplication of chromosome 16p11.2 [52]. In addition, ASD families are three to four times more likely to show de novo CNVs compared to control families [50]. However, it is important to note that not all of these CNVs may be associated with ASD since some of these alterations may have no phenotypic effect (benign CNVs) and can occur in the general population [50]. This area of research has been successful, however it is more expensive and time consuming than the two different approaches: genome-wide associations and candidate genes [53].

Three genome-wide association studies have found that individual common variants increase an individual’s risk of developing ASD. This approach involves studying all the variants across the whole genome to identify novel candidate genes and genetic contributors to common, complex diseases. These studies have evaluated single nucleotide polymorphisms (SNPs) and identified significant associations at
different loci [50]. In 2010, Anney et al. showed that SNP rs4141463, at 20p12.1 on the MACROD2 gene, was significantly associated [OR: 0.73; 95% CI: 0.66 – 0.82] with ASD case status [54]. Chromosome 3, which will be discussed further below, is the second area in the genome that has been implicated as a potential risk factor for ASD [47]. In contrast to the genome-wide association studies, this thesis used a candidate gene approach.

The candidate gene approach allows for an analysis of pre-specified genetic variants of interest and their associations with different conditions. With respect to ASD, the oxytocin receptor gene, located on chromosome 3, has been implicated in ASD (refer to Section 2.5 for elaboration), representing a potential gene of interest to evaluate for genetic variants. SNPs are the most common genetic variations within the genome, indicating that a different nucleotide than expected is present [55,56]. For example, a SNP could replace a cysteine (C) with a thymine (T) in a person’s DNA. This thesis examines the presence of SNPs because this approach has been proposed as a powerful means of identifying common variants, which underlie complex traits (i.e. social communication deficits), in a case-control design using family controls [50,53,55,56]. These genotypic differences can result in phenotypic variations that affect an individual’s physical characteristics, risk of some disease (i.e. ASD), and/or response to the environment [56].

2.4.3 Gene-Environment Interactions

Genetic factors (specifically different genotypes) can respond to environmental exposures in different ways through gene-environment interactions (GxE interaction) [33]. Three different hypotheses exist within this line of research. The first, known as the “epiphenomenon hypothesis,” argues that ASD, and associated prenatal, perinatal and postnatal issues are increased due to genetic factors, suggesting a potential genetic susceptibility to ASD [57]. Glasson et al. (2004) found that children with ASD encountered more prenatal and perinatal complications than their unaffected siblings, who in turn
experienced more complications than control subjects, indicating that the children with ASD may react differently to the same environmental stimuli [33,58,59]. For example, individuals with ASD were more likely to experience fetal distress [OR: 1.64; 95% CI: 1.15 – 2.34] compared to controls and were more likely to have been induced [OR: 1.40; 95% CI: 1.03 – 1.90] compared to their siblings [58]. The researchers did not address the temporality of this relationship. However, they suggest that the compromised prenatal experience for a genotype explains the presence of complications (i.e. fetal distress) and that this genotype leads to ASD [58].

The second hypothesis, “heterogeneity hypothesis,” proposes that each ASD case is the result of differential contribution of genetic and/or environmental factors [57]. It has been suggested that dysmorphic signs and cognitive impairments are worsened with a greater genetic contribution [57]. For example, Miles and Hillman (2000) found that greater genetic variations produce lower IQ and increase the number of minor physical anomalies in children with ASD [57,59].

The last hypothesis uses an epigenetic approach. This approach suggests that environmental factors interact with genetic factors and lead to a modification of the phenotypic expression of ASD [57]. Epigenetic studies in this area describe interactions between genetic variations, and oxidative stress and hypoxia [33,60-62]. This hypothesis was described after it was shown in animal models that neuroligin-deficient nematodes were hypersensitive to oxidative stress and MecP2-deficient mice were more susceptible to hypoxia [60,61]. These types of studies further hypothesized that altered synaptic genes could interact with environmental factors to increase the risk of ASD [60,61]. While three hypotheses have been formulated and researched, studies evaluating gene-environment interactions are low in power and rarely produce significant results. This is because not enough is known about the environmental and genetic factors separately [33].
2.5 Oxytocin and Autism Spectrum Disorder

While multiple rare and common genetic variants play roles in ASD, it has been suggested that each symptom cluster of the disorder, such as poor social communication, may be mediated by different genetic influences [30,63]. As individuals with ASD display impairments in social interaction and communication, they exhibit varying degrees of difficulties in social motivation and attention that causes an imbalance in attending to social stimuli, in social cognitive development and in social skills [30]. These behaviours can be improved through the administration of oxytocin in some cases [22,30,64]. In addition, three genome-wide association studies have found chromosome 3, where the oxytocin receptor gene is located, to be associated with ASD [47].

2.5.1 Oxytocin Neuropeptide and the Oxytocin Receptor

Oxytocin is a hormone. More specifically, it is a nine amino acid neuropeptide that is coded by the oxytocin gene. It is released into the bloodstream from the posterior pituitary gland after being synthesized in magnocellular neurons within the paraventricular and the supraoptic nuclei of the hypothalamus. Once in the bloodstream, oxytocin is circulated to different regions of the brain, where it exerts its functional effects after binding with its receptor [30,63,65,66].

The oxytocin receptor is the only known receptor that oxytocin can bind with. It is a 389 amino acid polypeptide, class I, G-protein coupled receptor with seven transmembrane domains that is encoded by the oxytocin receptor gene (OXTR). It is expressed in key areas of the brain: hippocampus [30,63,65,66], superior temporal gyrus [68,69], inferior frontal gyrus [68,69], amygdala and ventral striatum [30,63,65,66], as well as other brain regions to a lesser extent [30,63,65,66]. It is important to note that the amygdala is implicated in several aspects of social cognition and emotional processing, and the ventral striatum is the point in the brain where socially relevant pathways interconnect [66]. As a
result of where the oxytocin receptor is located, oxytocin is involved in many different aspects of human social behaviour including attachment, social recognition and aggression [30,65-67].

Oxytocin is responsible for numerous actions in the central and peripheral nervous systems [30,66]. Centrally, oxytocin is believed to be responsible for multiple social behaviours, including social recognition, social interactions, social responsivity, social motivation, and maternal bonding [63,66]. As a result of oxytocin’s central effects, abnormalities within this neuropeptide system have been investigated as a potential cause of different psychiatric disorders [70].

2.5.2 Oxytocin and Psychopathy

For decades oxytocin has been used in medicine, specifically to aid new mothers in parturition and lactation [70]. However, research has demonstrated that oxytocin administration in healthy subjects has the potential to improve ‘mindreading’ abilities [71], fear recognition [72], and trust [73,74] as well suggesting its efficacy in treating different psychopathological parameters, such as social attachment and cognition [70]. Thus, researchers have begun focusing on the use of oxytocin on cognition in psychiatric disorders (i.e. schizophrenia), depression and postpartum depression, and ASD [70].

In 1972, Bujanow showed that administration of oxytocin could reduce the psychotic symptoms seen in individuals with schizophrenia [75]. In follow-up studies, oxytocin administration was found to reduce the positive symptoms and improve ‘mindreading’ in individuals with schizophrenia [70]. In addition, oxytocin levels in blood serum have been evaluated with respect to schizophrenia and depression. These studies have shown that reduced oxytocin serum levels in individuals with schizophrenia were associated with a poorer ability to identify facial expressions [76] and the absence of trust-related interpersonal interactions [77] when compared to individuals without schizophrenia. Additionally, there is a potential worsening of symptoms in individuals with depression resulting from low oxytocin serum levels [78,79]. Furthermore, low oxytocin plasma concentrations in women are
indicative of an increased risk for postpartum depression (p < 0.05) [80]. These studies have suggested that oxytocin may be linked to numerous other psychiatric disorders.

In the past ten years, the focus has moved towards the effect of oxytocin on ASD symptoms and pathophysiology. Research has demonstrated that in healthy humans, low concentration levels of oxytocin are associated with lower social and cognitive functioning [65]. The administration of intranasal oxytocin has resulted in an increase in trust and cooperation among unrelated individuals with ASD, and in an improvement in the emotional recognition of facial expressions [30]. In individuals with ASD, plasma levels of oxytocin were found to be lower than that in healthy peers [20,65]. These results have prompted further investigation into the link between oxytocin and the social communication impairments displayed by individuals with ASD.

2.5.3 Neurobiology of Autism Spectrum Disorder and Oxytocin

Social-cognitive constructs are responsible for effective cooperation between individuals [69]. Four of these constructs, which when impaired are characteristic of different disorders, include emotion recognition, empathy/theory of mind, social reward seeking, and social communication [69]. Impairments in the social communication and social reward seeking constructs are characteristic of ASD. The oxytocin neuropeptide system, as described in section 2.5.1, is believed to affect cooperation motivation and behaviours [69]. Studies of children with ASD have identified macroscopic and microscopic abnormalities in their brain development when using structural and functional neuroimaging and neuropathological techniques [69].

Social reward processing refers to the seeking out and valuing of social interaction [69]. The salience brain network, the mesolimbic dopamine system, and areas within the ventral striatum and frontal lobes work together to create the motivation for social interaction [69]. The nucleus accumbens, which lies within the striatum, is involved in reward processing, and the ventromedial prefrontal cortex
and the anterior cingulate cortex are responsible for signalling and anticipating potential rewards [69]. Both of these areas express the oxytocin receptor gene (OXTR) and thus, oxytocin is able to bind there to exert its functional effects [30,63,65,66,68,69].

Functional neuroimaging studies have provided evidence that the structure and function of a subset of those brain regions involved in social reward processing are associated with the OXTR [69]. Tost et al. (2010, 2011) demonstrated that anterior cingulate cortex reactivity and volumetric differences are associated with OXTR [64,90]. In addition, positron emission tomography showed that dopamine levels within the striatum are associated with OXTR in female humans [81]. In animal studies, positron emission tomography demonstrated that oxytocin receptors are highly populated in the nucleus accumbens of prairie and meadow voles [82]. As such, individual differences in social reward processing may be associated with the OXTR [69].

The ability to perceive, understand and exchange information between individuals, known as social communication, involves different areas within the temporal and frontal lobes [68,69]. Verbal and non-verbal social cues are processed by the superior temporal gyrus [69,83]. The primary auditory cortex, found within the superior temporal gyrus, breaks down verbal communication signals [83]. Speech is produced in the inferior frontal gyrus and the premotor cortex [84]. Effective social communication occurs when these areas are working together (referred to as the social communication neural system) [69].

Magnetic resonance imaging has demonstrated that the OXTR may be implicated in ensuring the proper functioning of the social communication neural system [69]. While no direct link between OXTR and the mechanisms underlying social communication has been found, Riem et al. (2011, 2012) showed greater left inferior frontal gyrus reactivity to children crying in parents who received intranasal oxytocin administration [69,85]. Furthermore, left superior temporal gyrus activation depends on OXTR
methylation status [86] and this activation is reduced in individuals with ASD who have poorer language outcomes [87]. In 2011, Tops et al. found that vocal communication and information comprehension are associated with OXTR [88]. While, it remains unknown which and how OXTR polymorphisms affect these areas in their structure and function, research has implicated these polymorphisms in the severity of communication deficits in ASD [1,19,20,64,69].

2.5.4 Oxytocin, Autism Spectrum Disorder and Social Communication Deficits

Since oxytocin is involved in modulating social behaviour, the OXTR has become a strong functional candidate gene that may be involved in the development of ASD [64]. SNPs within the OXTR have been seen to contribute to the ‘asociality’ in individuals with ASD [19,22,64].

Five previous preliminary studies have examined different SNPs within the OXTR among children with ASD, specifically the presence of SNPs at marker rs2254298 and to a lesser extent marker rs53576 [1,19,20,89,94]. In a family study of Caucasians by Jacob et al. (2007), the potential rs2254298G risk allele was found to be significantly associated with a diagnosis of ASD [20]. A Chinese case-control study by Wu et al. (2005) found that the rs2254298A and rs53576A risk alleles were significantly associated with a diagnosis of ASD [19]. Homozygosity for the risk allele rs53576A has also been found to be associated with low task-related amygdala activations, indicating deficits in empathy and attachment [90-92].

Campbell et al. (2011), on the other hand, did not find these two SNPs to be significantly associated with ASD. Instead, they found that the rs2268493, rs1042778 and rs7632287 markers were significantly associated with ASD [64,93]. Campbell et al. (2011) represents the largest family-based study, with more than 2,000 cases of ASD, to have investigated this relationship. This study showed the rs2268493T, rs1042778G and rs7632287G risk alleles were significantly associated not only with case status but also with categorical phenotypic cutoff scores on the ADI-R [64]. This is also the first study to
show that OXTR genetic polymorphisms are associated with the social aspects of ASD [64]. It should be noted however that Campbell et al. (2011) were unable to find significant results for any of the SNPs being evaluated after accounting for multiple comparisons in their study. This may be due to the low power that could be improved by using a larger sample size or evaluating fewer SNPs.

The markers implicated by the Campbell et al. (2011) study have also been previously shown to be significantly associated with ASD [21,89,94]. A study using Irish families (N = 179) found the rs7632287A marker to be significantly associated with case status [89]. Furthermore, the rs1042778G marker was associated with a DSM-IV diagnosis of ASD in a sample of 152 Israeli subjects prior to correcting for multiple comparisons [94]. Finally, the rs2268493T marker was significantly associated with Asperger’s disorder in 530 Caucasian individuals [21]. More research is needed to determine the relationship between OXTR and ASD, as previous results have been inconclusive [1,19,20,22,64,89-94].

2.5.5 Functional Impact of the Four OXTR Variants

The functional impact of these variants in vivo has not been extensively studied. Two of these variants (rs53576 and rs2254298) are located in the intron 3 region of the OXTR [64]. This region represents a short area of non-coding found within genes [95]. The functional impact of these variants remain unknown, however, a point mutation in this area could alter the reading frame of the RNA strand resulting in an inhibition of splicing activity or functional connectivity [96]. For example, Wang et al. (2013) found that an AA genotype at OXTR rs53576 is significantly associated with a reduction in the local functional connectivity density in the hypothalamus [96]. In addition, the resting-state functional connectivity between the hypothalamus and left dorsolateral prefrontal cortex was weaker in individuals with an AA genotype at this marker [96].

The rs7632287 marker is located in the intergenic region of the OXTR [64]. This is a region of non-coding that may act to control nearby genes [97]. The functional impact of mutations within this area
remains largely unknown [97]. It is believed that mutations in the area can affect the higher-order structures produced by the gene [98]. If this is true, a potential impact of mutations at this location within the OXTR is the production of a OXTR receptor that oxytocin will be unable to bind to. This would result in a reduction of oxytocin within the bloodstream.

Finally, the rs1042778 marker is located within the exon 4-3’ UTR region of the OXTR [64]. This region represents an area of coding in the RNA strand [95]. Mutations in this location can alter translation and mRNA localization that would affect the level of protein expressed, and can also create a new start codon [95]. With respect to the rs1042778 marker, the expression of a ‘T’ allele has been associated with a reduction in oxytocin plasma levels [99]. Feldman et al. (2012) suggest that regulating the oxytocin signaling pathway and/or peripheral oxytocin activity is the functional of this genetic marker [99]. This study represents the only known attempt to determine the functional effect of OXTR polymorphisms at this marker. The authors also state that more molecular studies are needed to determine the functional impact of these four OXTR variants.

2.6 Conclusion

In conclusion, oxytocin has been shown to be involved in mediating different social behaviours in humans. Impairments within this neuropeptide system have been associated with several psychiatric disorders, including ASD, yet the relationship between oxytocin and ASD has yet to be determined. The social interaction and communication deficits of ASD have been associated with changes in oxytocin functioning with specific focus on OXTR variants [19-21,30,64,65,89]. However, research implicating risk alleles supplied by the presence of SNPs rs2254298, rs2268493, rs7632287, rs1042778 and rs53576 in the development of social communication deficits seen in ASD has been inconclusive. Only one study, by Campbell et al. (2011), attempted to show associations between OXTR genotypes and different phenotypic domains of ASD. Ethnicity has previously been implicated as playing a role in the potential
association between OXTR and ASD, with different alleles acting as risk inducing in Caucasian and Asian populations. For example, the ‘G’ and ‘A’ alleles provided by the rs2254298 marker confer risk in Caucasian and Asian populations respectively [19,20]. Thus, the purpose of this thesis is to provide further insights into the role of SNPs in the oxytocin neuropeptide system on the risk of ASD and on resulting social communication deficits among a multi-ethnic sample.
2.7 References


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Chapter 3

General Methods

3.1 Objectives

The purpose of this study is to contribute to the existing knowledge of the aetiology of autism spectrum disorder (ASD) and specifically the association of single nucleotide polymorphisms (SNPs) rs2254298, rs7632287, rs1042778 and rs53576 in the oxytocin receptor gene (OXTR) with ASD, through two empirical objectives:

1. To compare the genotype distributions of four OXTR variants in individuals with ASD to those in their unaffected siblings, and
2. To determine the association between specific OXTR genotypes and greater social communication deficits among individuals with a diagnosed ASD.

3.2 Study Population

This study used data from the Autism Spectrum Disorders – Canadian-American Research Consortium (ASD-CARC) Research Registry [1]. This registry was formed in 2001 by Dr. Jeanette Holden (Principal Investigator) and is currently run by Dr. Xudong Liu (Program Director). The ASD-CARC, based in Kingston, Ontario, is a multi-disciplinary team of more than 70 researchers and clinicians working with more than 3000 families internationally [1]. Participation is voluntary, with individuals being referred to the Registry’s website by their physicians or other support resources, and/or individuals hearing of the site from family members or friends [1]. Individuals within the Registry are invited to complete online questionnaires about family characteristics (i.e. ethnicity), outcomes (i.e. diagnoses of ASD and/or other co-morbid disorders), and exposures [1]. As a result of how the data are obtained, there is a high chance of missing data. To reduce missing data, the ASD-CARC is in constant contact with the Registry’s
families reminding them to complete the questionnaires and seeking clarification by phone [1]. A subset of the ASD-CARC registry families with DNA samples for OXTR genotyping was used totaling 1299 participants. Appendix A contains a flowchart outlining the number of potential participants for each objective following the application of the different eligibility criteria.

### 3.3 Genotyping Procedure

This thesis, and associated manuscripts, used a genotyping procedure, known as the TaqMan SNP Genotyping, as the sole exposure measurement. This approach has been used in many large-scale case-control association studies because it is an advanced, mature and validated technology [2]. *Table 3.1* describes five SNPs of interest at the outset based on the literature review: rs2254298, rs2268493, rs7632287, rs1042778 and rs53576 [3-8].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>SNP</th>
<th>Base-Pair Position</th>
<th>Alleles</th>
<th>Minor Allele Frequency*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXTR</td>
<td>3</td>
<td>rs2254298</td>
<td>8760542</td>
<td>G/A*</td>
<td>0.2071</td>
<td>Jacob et al. (2007); Wu et al. (2005)</td>
</tr>
<tr>
<td>OXTR</td>
<td>3</td>
<td>rs2268493</td>
<td>8759154</td>
<td>T/C*</td>
<td>0.2049</td>
<td>Campbell et al. (2011); DiNapoli et al. (2014)</td>
</tr>
<tr>
<td>OXTR</td>
<td>3</td>
<td>rs7632287</td>
<td>8749760</td>
<td>G/A*</td>
<td>0.2400</td>
<td>Campbell et al. (2011); Tansey et al. (2009)</td>
</tr>
<tr>
<td>OXTR/CAV3</td>
<td>3</td>
<td>rs1042778</td>
<td>8752859</td>
<td>G/T*</td>
<td>0.4109</td>
<td>Campbell et al. (2011); Lerer et al. (2008)</td>
</tr>
<tr>
<td>OXTR</td>
<td>3</td>
<td>rs53576</td>
<td>8762685</td>
<td>G/A*</td>
<td>0.3894</td>
<td>Wu et al. (2005)</td>
</tr>
</tbody>
</table>

* indicates minor (risk) allele
+ minor allele frequency (MAF)
^ MAF numbers were obtained from the NCBI dbSNP database [9]

The five polymorphisms were genotyped using validated custom TaqMan SNP Genotyping Assays (http://www.lifetechnologies.com/) on an ABI Prism ViiA7 Real-Time PCR machine, using 384-well plates, with the conditions outlined in Appendix B. Duplicate samples and negative controls were
included in each plate to check the accuracy of genotyping. Genotypes were then automatically scored with the Sequence Detection System (SDS) Viia7 (Version 1.2) software using standard parameters. The only change made to standard parameters was that a quality score of 80 was used instead of 95. The quality score was changed because at 95 many samples were being discarded even though they were clearly a specific genotype. The SDS creates a plot based on the fluorescence signals from each well to indicate which alleles are present in the sample [2]. Figure 3.1 shows the SDS plot from one plate of the rs53576 marker. With this information it is then possible to determine the allele and genotypic distribution within the sample.

![Allelic Discrimination Plot](image)

**Figure 3.1** A SDS generated allelic discrimination plot from a single 384-well plate for rs53576. The blue dots represent individuals who are homozygous for the G allele (GG), the green dots represent heterozygous individuals (AG), and the red dots represent individuals who are homozygous for the A allele (AA). The 'x' represents individuals with undetermined genotypes who were re-genotyped at a later date.

Following a sample of genotyping ($N_{\text{plate}} = 1$) it was decided that a single marker, rs2268493, be dropped due to its rarity (see Figure 3.2). Within the sample of a single plate, only a single person
displayed a different genotype than being heterozygotes (CT) within the rs2268493 marker. The allele and genotype distributions would not allow us to compare between groups. Thus, only four SNPs were genotyped and included in the analyses.

![Allelic Discrimination Plot](image)

*Figure 3.2* A SDS generated allelic discrimination plot from a single 384-well plate for rs2268493. The green dots represent individuals who are homozygous for the T allele (TT) and the red dot represent an individual who is homozygous for the C allele (CC). The ‘x’ represents individuals with undetermined genotypes who were re-genotyped at a later date.

3.4 Objective One

The first objective of this thesis was to compare twelve OXTR genotypes, from four SNPs, in individuals with ASD to that in their unaffected siblings using a case-control approach.

3.4.1 Participants

The cases and controls from this study were all obtained from the ASD-CARC Research Registry. Cases were eligible if they had a confirmed diagnosis (by clinician) of ASD without a known genetic disorder (i.e. excluding karyotyping-detectable chromosomal anomalies and Fragile X syndrome), were from a
simplex (SPX) family (only family member with a diagnosis of ASD), did not have other neurodevelopmental disabilities (i.e. ADHD), were older than three, and had an unaffected sibling in the registry. Controls were those unaffected siblings who were older than three at the time that genetic samples were obtained.

Cases and their unaffected sibling formed a 1:1 sibling-pair matching that produced a total sample size of 1214 participants. A single control was randomly selected in situations where there were multiple controls eligible. From those remaining eligible controls, an additional sibling was marked as an alternative sibling and was subsequently genotyped to be used in circumstances where the original sibling control failed genotyping (i.e. did not produce a genotype). Of the 43 individuals marked as alternative siblings, five were used in the analyses instead of the originally selected control. Table 3.2 outlines the total sample size for each SNP that accounts for genotyping failure.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Total Failures</th>
<th>Who Failed in the Sibling-Pair</th>
<th>Total Removed</th>
<th>Final Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2254298</td>
<td>15</td>
<td>Both: 3 Case: 4 Control: 5</td>
<td>24</td>
<td>1190</td>
</tr>
<tr>
<td>rs1042778</td>
<td>24</td>
<td>Both: 3 Case: 5 Control: 13</td>
<td>42</td>
<td>1172</td>
</tr>
<tr>
<td>rs53576</td>
<td>17</td>
<td>Both: 3 Case: 6 Control: 5</td>
<td>28</td>
<td>1186</td>
</tr>
<tr>
<td>rs7632287</td>
<td>49</td>
<td>Both: 3 Case: 22 Control: 21</td>
<td>90</td>
<td>1124</td>
</tr>
</tbody>
</table>

### 3.4.2 Outcome Assessment

This study only used a single outcome measure: ASD. ASD was treated as a dichotomous variable (yes, no). Individuals were considered as having ASD, and subsequently classified as cases, if they met the diagnostic criteria set out by the *Diagnostic and Statistic Manual of Mental Disorders* (DSM). This
information was already provided at the time the individual entered into the study through a letter of diagnosis from a clinician.

3.4.3 Covariates

It is deemed unlikely that the genetic variants within the OXTR of an individual are related to any external factors, thus limiting the potential confounders in the relationship between OXTR SNPs and the risk of ASD. Data surrounding two potential covariates, the sex of the participants (male, female) and ethnicity (Caucasian, Asian, Black, First Nations, Other), were collected by questionnaire and recorded at the ASD-CARC. Previous research has adjusted for sex in their analyses due to the large sex ratio seen in ASD and therefore, this variable was evaluated for confounding [10]. It has also been demonstrated that the risk alleles for specific markers differ based on ethnicity due to population genetics [3-8].

3.5 Objective Two

The second objective was to evaluate and describe the association between specific OXTR genotypes and resulting social communication deficits in individuals with a diagnosed ASD using a cross-sectional approach.

3.5.1 Participants

Participants for this objective were selected from the ASD-CARC Research Registry. Eligibility criteria remained the same as objective one except that the participants were no longer required to have a sibling in the registry but rather were required to have an ADI-R score. There were approximately 275 participants who met these criteria. Table 3.3 outlines the total sample size for each SNP that accounts for genotyping failure.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Total Failures</th>
<th>Final Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2254298</td>
<td>1</td>
<td>274</td>
</tr>
<tr>
<td>rs1042778</td>
<td>1</td>
<td>274</td>
</tr>
<tr>
<td>rs53576</td>
<td>1</td>
<td>274</td>
</tr>
<tr>
<td>rs7632287</td>
<td>6</td>
<td>269</td>
</tr>
</tbody>
</table>
This study used a single outcome measure: a component of the *Autism Diagnostic Interview-Revised* (ADI-R). The ADI-R is a standardized, investigator-based interview that seeks information related to the criteria required for a DSM-IV diagnosis of ASD [12,13]. It includes 93 items each scored between zero and three, which have been categorized into three domains of functioning to align with the ‘triad of impairments’ described in the DSM-IV: communication, reciprocal social interactions, and restrictive and obsessive behaviours [13,14]. The communication and reciprocal social interactions categories include questions on speech development and if the child is able to use the appropriate word within a conversation, and how the child interprets emotional responses and interacts with others respectively [13]. These questions are used to assess the social interaction and communication domain of the DSM-V. The final category, which was designed to evaluate the restrictive and repetitive behaviour domain, includes questions surrounding behaviours that are stereotypically associated with ASD (i.e. item fixation and phrase repetition) [13].

The component of the ADI-R that is of interest for this thesis is the communication category. This category is used to generate social communication scores. There are 41 questions on the ADI-R that deal with both verbal and non-verbal communication [13,14]. For example, it asks whether or not there is a delay or total lack of language not compensated by gesture or if there is relative failure to initiate or sustain conversational interchange, etc. [13,14]. Appendix C provides the scoring scheme for the ADI-R. To estimate the severity of autistic symptoms, researchers have used overall scores or scores in specific domains [14]. Since oxytocin is implicated in social behaviours, the social communication scores were used as the sole outcome of interest from the ADI-R.

Typically, the ADI-R social communication score is dichotomized using a standard cutoff score of greater than or equal to eight for verbal subjects and greater than or equal to seven for non-verbal
subjects to meet the minimum score for this domain according to the DSM-IV [14]. However, for the purpose of this study, the ADI-R social communication score was treated as a continuous variable to examine the association between the degree of social communication deficits and the four OXTR variants. *Figure 3.3* shows the distribution of social communication scores on the ADI-R from a previously published subset of verbal individuals (N = 339) within the ASD-CARC [15]. This plot shows that the social communication score, which carries a maximum score of 26 for verbal participants, ranges from approximately two to 22. It is because of this figure that the current investigators decided to treat the variable as continuous *a priori* as the scores on the y-axis approximately range from one to 22. The ADI-R has been shown to have high inter-rater and test-retest reliability, and internal validity [13,14].

![Figure 3.3](image)

*Figure 3.3* A correlation plot between communication score and age at assessment in a subset of ASD-CARC verbal participants (N = 339) directly replicated from McGarry (2013) [15].

### 3.5.3 Covariates

As the exposure variable for this objective is also genetic, it is deemed unlikely that many factors will be independently associated with the exposure and therefore, represent ‘true’ confounders. Variations in administration of the ADI-R were included as covariates. These variations include: age at ADI-R
administration (<= 11 years, > 11 years), interviewee relationship (mother, father, both), how the ADI-R was administered (telephone, face-to-face) and sex (male, female). Since the maximum ADI-R social communication scores differs for verbal and non-verbal individuals, the analyses were stratified by communication status [14].

3.6 Statistical Analysis

3.6.1 Descriptive Analysis

For both objectives, key characteristics of the study population (sex and ethnicity) were examined using descriptive statistics. For objective two, age of the child at ADI-R administration, communication status of the child (verbal and non-verbal), version of ADI-R used, interviewee relationship, and how the ADI-R was administered were also presented. Frequency tables were generated for the categorical variables: sex, ethnicity, age at ADI-R administration, version of ADI-R used, interviewee relationship, administration type, and communication status. For objective one, the cases and controls were compared using Chi-square tests or Fisher’s exact tests. For the second objective, means and standard deviations for the ADI-R social communication scores were calculated. All statistical analyses were performed using SAS 9.4 (SAS, Institute, Cary, NC).

3.6.2 Hardy-Weinberg Equilibrium

Prior to analyzing the data for this thesis, the Hardy-Weinberg equilibrium (HWE) for genotype frequency distribution was assessed in the unaffected participants (sibling controls). The law of Hardy-Weinberg states that the amount of genetic variation within a population will remain constant from one generation to another if it is not affected by other factors resulting in disequilibrium. In genetic association studies this deviation can result from either population genetic causes or the presence of a genetic association. The latter is of particular importance for this thesis. The Chi-square goodness-of-fit test was used to assess HWE using the HWE program for two alleles [16-18]. The null hypothesis was
that the genotype distribution is in HWE while the alternative hypothesis was that it is in disequilibrium. The significance level for HWE is arbitrary, with the alpha levels typically ranging from 0.001 to 0.05 [17]. For this thesis, it was decided *a priori* that if \( p < 0.001 \), the null hypothesis would be rejected. In practice, the aim is to fail to reject the null hypothesis and say that the genotype distributions of the specific markers are in HWE [18].

If the null hypothesis is rejected, it indicates that the genotype distribution is deviating from HWE. Deviations from HWE can be the result of four different, but not mutually exclusive, reasons: inbreeding, genetic drift, population bottlenecks, and founder events [16,18,19]. Inbreeding changes genotypic proportions resulting in an increase in homozygote frequencies and a decrease in heterozygotes since related parents are more likely to give the same alleles to their offspring [19]. The amount of fluctuation of allele frequency from one generation to the next, referred to as genetic drift, increases in small populations resulting in the loss of variety (polymorphisms) in the genome [19]. This may reduce genetic diversity [19]. Population bottlenecks occur when there is a severe decrease in population size that further reduces genetic diversity and increases the effect of genetic drift [19]. Finally, people migrate from one habitat to another resulting in rapid changes in allele frequency, known as founder effects, and high potential for divergence and speciation [19].

### 3.6.3 Oxytocin Receptor Genotypes and Risk of Autism Spectrum Disorder

For the first objective, a conditional logistic regression analysis in an additive genetic model was used to evaluate the relationship between OXTR genotypes and ASD, using SAS (Version 9.4, SAS Institute, Cary, NC). The effects of potential confounders on the relationships between genotypes and ASD were assessed using backwards elimination, with an exclusion p-value criterion of \( \alpha = 0.05 \), from the fully adjusted model. The final model was generated using a change-in-estimate approach that retains confounders in the model if their removal alters the odds ratio by ten percent or greater.
The Benjamini-Hochberg adjustment [20] is a post-hoc test that was used to evaluate if multiple comparisons were an issue in this study. This method provides corrected p-values by controlling for the expected false discovery rate across the four different SNPs [16]. It produces conservative p-values because it assumes independence of tests being conducted [16]. This is a conservative approach because it is highly unlikely that these SNPs are independent of one another, especially when previous studies have demonstrated these markers to be in linkage disequilibrium (LD) with one or more OXTR variants [3,5]. LD refers to the non-random association of alleles at different loci (different markers). If the p-value is less than 0.05, the associations described above will be considered significant. All analyses were computed using SAS.

3.6.4 Oxytocin Receptor Genotypes and Social Communication Deficits

For the second objective, the outcome of interest was the ADI-R social communication score. A linear regression in an additive genetic model was used to evaluate the differences in ADI-R social communication scores across different OXTR genotypes, while adjusting for sex, the age at the time of ADI-R administration, interviewee relationship, and interview format. A linear regression approach was taken because the social communication scores treated as continuous followed a normal distribution. The effect of potential covariates and degree of social communication deficit were assessed in verbal and non-verbal participants using backwards elimination, with an exclusion p-value criterion of $\alpha = 0.05$, from the fully adjusted model. The final model was generated using a change-in-estimate approach that retains confounders in the model if their removal alters the parameter estimates by ten percent or greater. If the p-value is less than 0.05, the associations described above will be considered significant. Non-verbal analyses are reported in Chapter 6. All analyses were computed using SAS.
3.6.5 Sensitivity Analyses

Sensitivity analyses were conducted to examine whether the presence of mixed ethnicities within the samples of both objectives was masking any effect. As a result, the analyses for the first and second objectives were done a second time in a sub-sample of 305 and 159 Caucasian only participants respectively. In addition, within objective two, the analyses were carried out in a sample restricted to mothers only and telephone interviews only to examine whether or not the relationships found in the complete sample remained. These restricted analyses are reported in Chapter 6.

3.6.6 Linkage Disequilibrium Calculation

Linkage disequilibrium (LD) across the four OXTR SNPs was investigated using Haploview. LD refers to the non-random association of alleles at different loci (different markers). $r^2$ values for each combination pair of SNPs were used to measure LD. A score of 1 indicates that the two markers provide identical information [21]. Appendix D provides the results from the LD analyses.

3.7 Ethical Considerations

The creation and maintenance of the ASD-CARC Research Registry has been reviewed and granted approval by the Queen’s University Health Sciences Research Ethics Board. Expedited ethics approval was granted to the candidate from the Queen’s University Health Sciences Research Ethics Board for this thesis (Appendix E) for use of the data. No individual data or names from the registry were made available to the student and all files were stored in a secure, locked office at the ASD-CARC.
3.8 References


Chapter 4

Genetic variation in the oxytocin receptor gene and risk of autism spectrum disorder

Meagan Milton, BSc, MSc (Candidate); Hélène Ouellette-Kuntz, PhD; Melissa Hudson, BSc;
Amy McNaughton, PhD; Xudong Liu, PhD

Formatted for *The American Journal of Human Genetics*

4.1 Abstract

Autism spectrum disorder (ASD) affects approximately one percent of the population, making it one of the leading childhood disorders. It is a multifactorial condition resulting from either environmental or genetic factors or a combination of these. Oxytocin is a neuropeptide implicated in ASD due to its involvement in human social behaviours. Oxytocin nasal sprays have been used to promote stronger and more appropriate social behaviour and affect in individuals with ASD. The oxytocin receptor gene (OXTR) encodes the oxytocin receptor through which the neuropeptide is able to exert its functional effects. Recent reports have suggested that single nucleotide polymorphisms (SNPs) within the OXTR may be associated with ASD. The purpose of this study was to evaluate the association between the four OXTR variants, and ASD in 607 simplex (SPX) families. Cases were compared to their unaffected siblings using a conditional logistic approach. These comparisons demonstrated a significant weak negative association between the AG genotype of the rs53576 marker and ASD. This protective effect was not replicated in a Caucasian only sample. Future studies should focus on examining haplotype formation between these SNPs in large sample sizes.
4.2 Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects approximately one percent of the population [1]. It is characterized by impairments in two main domains: (1) social interaction and communication, and (2) restrictive behaviour [2-4]. The aetiology of ASD is multifactorial with genetic and environmental factors playing a role [5,6]. Research indicates that two to three percent of families are multiplex providing further support of a genetic role in ASD aetiology [7]. Furthermore, families with a single child with ASD have a four and seven percent reoccurrence rate if the second child is male or female respectively [5]. Evidence of a strong genetic link has also been demonstrated by twin studies that estimate the heritability of ASD at between 60 to 90 percent [8-11]. While research has implicated a genetic component in the aetiology of ASD, it remains unclear where in the genome mutations arise that lead to this disorder.

Multiple genetic variants have been shown to play a role in ASD. It has been suggested that each symptom cluster, such as poor social communication, may be mediated by different genetic influences, such as oxytocin [9,12]. Preliminary studies have demonstrated the presence of single nucleotide polymorphisms (SNPs) within the oxytocin receptor gene (OXTR) in children with ASD. In a study by Wu et al. (2005), OXTR rs2254298A and rs53576A risk alleles were significantly associated with ASD in Japanese population [2]. Jacob et al. (2007), on the other hand, identified the rs2254298G risk allele in the OXTR as being the only SNP significantly associated with a diagnosis of ASD in a study of Caucasian children [3]. More recently, Campbell et al. (2011) examined 25 different SNPs in the largest study to evaluate the relationship between OXTR and ASD. They found the rs2268493T, rs1042778G and rs7632287G markers, rather than the two previously mentioned, to be significantly associated with ASD [4].
Taken together, these results indicate that social interaction and communication deficits of ASD may be associated with changes in oxytocin functioning through mutations in the OXTR [2,3,12]. However, research in this area has provided conflicting reports of OXTR SNPs that could in part be attributed to lack of power. Further studies should either use larger sample sizes or fewer SNPs to improve study power. To identify OXTR SNPs associated with ASD, the current study examined twelve OXTR genotypes produced from four different SNPs (rs2254298, rs7632287, rs1042778, and rs53576). The genotype distributions of four OXTR variants in individuals with a diagnosed ASD were compared to that in their unaffected siblings.

4.3 Material and Methods

4.3.1 Study Population

A subset of the Autism Spectrum Disorders – Canadian-American Research Consortium (ASD-CARC, www.autismresearch.com) registry families was selected for this study. Participants were considered cases (n = 607) if they had a confirmed diagnosis (by clinician) of ASD in the absence of a known aetiology (i.e. excluding karyotype-detectable chromosomal anomalies and Fragile X syndrome) or other neurodevelopmental disabilities (i.e. Down syndrome), were from a simplex (SPX) family (only family member with a diagnosis of ASD), were older than three at the time that genetic samples were obtained, and had an unaffected sibling in the Registry. Controls (n = 607) were those unaffected siblings that had been genotyped and were older than three at the time that genetic samples were obtained. A single control was randomly selected in situations where there were multiple controls eligible. Cases and their unaffected sibling formed a 1:1 sibling-pair that produced a total sample size of 1214 participants. Ethics approval for this study was obtained from the Queen’s University Health Sciences Research Ethics Board.
4.3.2 Data Collection

Online questionnaires are available at the ASD-CARC website (http://asdcarc.com/). These questionnaires provide information surrounding the participants’ diagnosis, ASD characteristics, and different family characteristics. Families have the option of completing these questionnaires for children who do not have ASD as well.

Saliva samples were obtained from the sibling-pairs. Genotyping of the four SNPs (rs2254298, rs7632287, rs1042778 and rs53576) was carried out using validated custom TaqMan SNP Genotyping Assays (http://www.lifetechnologies.com) on an ABI Prism ViiA7 Real-Time PCR machine, using 384-well plates. Duplicate samples and negative controls were included in each plate to check the accuracy of genotyping. Genotypes were then automatically scored with the Sequence Detection System (SDS) ViiA7 (Version 1.2) software using standard parameters.

4.3.3 Statistical Analysis

Hardy-Weinberg equilibrium (HWE) was evaluated in the controls prior to data analysis using the HWE program for two alleles [13,14]. The relationship between the four SNPs (rs2254298, rs7632287, rs1042778 and rs53576) and ASD was examined using a conditional logistic regression in an additive genetic model approach. Sex was the only potential confounder evaluated using a change-in-estimate approach with a cut-off of ten percent. The Benjamini-Hochberg adjustment was used to control the false discovery rate. False discovery adjusted odds ratios, and associated 95 percent confidence intervals, are reported for each SNP. A corrected p-value of < 0.05 indicated statistical significance. All analyses were conducted using SAS (Version 9.4, SAS Institute, Cary, NC).

4.4 Results

Males comprised 56 percent of the sample. The mean age at the time of data extraction was 21.65 ± 7.81 (5 – 71) for the cases and was 21.74 ± 8.40 (6 – 67) for controls. Tables 4.1 and 4.2 summarise the allele
and genotype frequencies in individuals with ASD versus their unaffected siblings for the rs2254298, rs1042778, rs53576, and rs7632287 markers. These distributions did not deviate from HWE.

Overall, ASD status did not significantly differ based on the OXTR SNPs studied after adjusting for false discovery rate. Table 4.3 provides analyses for each marker prior to and after adjusting for false discovery rate. Only one genotype, AG from the rs53576 marker, was significantly associated with a decreased risk of ASD when compared to the reference GG genotype (OR = 0.707, 95% CI: 0.512 – 0.975, p_OR = 0.0582). However, after adjusting for false discovery rate, the association was no longer significant. The other genotype from the rs53576 marker, AA, was not associated with ASD (AA: OR = 0.955, 95% CI: 0.544 – 1.677) when compared to the reference genotype (GG). No associations, prior to and after adjusting for false discovery rate, were seen when restricting the sample to Caucasians only.
### Table 4.1 Allele and genotype distributions of OXTR rs2254298, rs53576 and rs7632287 in children with ASD and their unaffected siblings in the complete (mixed) sample

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele</th>
<th>Genotype</th>
<th>HWE</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>G</td>
<td>A</td>
<td>N</td>
</tr>
<tr>
<td>rs2254298</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1190</td>
<td>1018</td>
<td>172</td>
<td>595</td>
</tr>
<tr>
<td>(85.5)</td>
<td>(14.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>1190</td>
<td>1005</td>
<td>185</td>
<td>595</td>
</tr>
<tr>
<td>(84.4)</td>
<td>(15.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs53576</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1186</td>
<td>810</td>
<td>376</td>
<td>593</td>
</tr>
<tr>
<td>(68.3)</td>
<td>(31.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>1186</td>
<td>820</td>
<td>366</td>
<td>593</td>
</tr>
<tr>
<td>(69.0)</td>
<td>(31.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7632287</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1124</td>
<td>871</td>
<td>253</td>
<td>562</td>
</tr>
<tr>
<td>(77.5)</td>
<td>(22.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>1124</td>
<td>868</td>
<td>256</td>
<td>562</td>
</tr>
<tr>
<td>(77.2)</td>
<td>(22.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*the numbers in brackets represent percentages

### Table 4.2 Allele and genotype distribution of OXTR rs1042778 in children with ASD and their unaffected siblings in the complete (mixed) sample

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele</th>
<th>Genotype</th>
<th>HWE</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>G</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>rs1042778</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1172</td>
<td>712</td>
<td>460</td>
<td>586</td>
</tr>
<tr>
<td>(60.8)</td>
<td>(39.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>1172</td>
<td>729</td>
<td>443</td>
<td>586</td>
</tr>
<tr>
<td>(62.2)</td>
<td>(37.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*the numbers in brackets represent percentages
### Table 4.3 Sex-adjusted odds ratios for the associations between OXTR SNPs and ASD in the complete (mixed) and Caucasian only samples

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>N</th>
<th>Genotype</th>
<th>Odds Ratio (95% CI)</th>
<th>p&lt;sub&gt;a&lt;/sub&gt;</th>
<th>p&lt;sub&gt;b&lt;/sub&gt;</th>
<th>N</th>
<th>Genotype</th>
<th>Odds Ratio (95% CI)</th>
<th>p&lt;sub&gt;a&lt;/sub&gt;</th>
<th>p&lt;sub&gt;b&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXTR</td>
<td>rs2254298</td>
<td>595</td>
<td>AA</td>
<td>1.497 (0.654, 3.429)</td>
<td>0.4328</td>
<td>0.8808</td>
<td>305</td>
<td>AA</td>
<td>1.559 (0.557, 4.362)</td>
<td>0.2988</td>
<td>0.7528</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AG</td>
<td>1.180 (0.823, 1.691)</td>
<td>0.8808</td>
<td>0.8808</td>
<td></td>
<td>AG</td>
<td>0.846 (0.524, 1.366)</td>
<td>0.2084</td>
<td>0.7528</td>
</tr>
<tr>
<td>OXTR</td>
<td>rs7632287</td>
<td>562</td>
<td>AA</td>
<td>0.764 (0.375, 1.558)</td>
<td>0.5183</td>
<td>0.8808</td>
<td>284</td>
<td>AA</td>
<td>0.860 (0.332, 2.231)</td>
<td>0.7702</td>
<td>0.9613</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AG</td>
<td>0.916 (0.635, 1.323)</td>
<td>0.8314</td>
<td>0.8808</td>
<td></td>
<td>AG</td>
<td>0.974 (0.586, 1.620)</td>
<td>0.8729</td>
<td>0.9613</td>
</tr>
<tr>
<td>OXTR/CAV3</td>
<td>rs1042778</td>
<td>586</td>
<td>TT</td>
<td>0.988 (0.622, 1.569)</td>
<td>0.2740</td>
<td>0.8808</td>
<td>298</td>
<td>TT</td>
<td>0.898 (0.479, 1.682)</td>
<td>0.9613</td>
<td>0.9613</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GT</td>
<td>0.846 (0.614, 1.165)</td>
<td>0.7328</td>
<td>0.8808</td>
<td></td>
<td>GT</td>
<td>0.785 (0.491, 1.682)</td>
<td>0.3764</td>
<td>0.7528</td>
</tr>
<tr>
<td>OXTR</td>
<td>rs53576</td>
<td>593</td>
<td>AA</td>
<td>0.955 (0.544, 1.677)</td>
<td>0.6311</td>
<td>0.8808</td>
<td>302</td>
<td>AA</td>
<td>0.538 (0.234, 1.238)</td>
<td>0.1932</td>
<td>0.7528</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AG</td>
<td>0.707 (0.512, 0.975)</td>
<td>0.0582</td>
<td>0.4656</td>
<td></td>
<td>AG</td>
<td>0.814 (0.520, 1.273)</td>
<td>0.6758</td>
<td>0.9613</td>
</tr>
</tbody>
</table>

a indicates unadjusted p-value

b indicates FDR-adjusted p-value
4.5 Discussion

ASD is one of the most common disorders in children [1]. Researchers have begun looking at multiple avenues that may result in the presence of one or more of the different symptom clusters commonly associated with ASD [9,12]. Research has demonstrated that individuals with ASD display lower plasma levels of oxytocin compared to their healthy peers [15]. With respect to social communication deficits seen in individuals with ASD, oxytocin nasal sprays have been used to promote stronger and more appropriate social behaviour and affect in individuals with ASD [16]. These two findings have indicated a potential link between oxytocin and ASD, with much of the current research focus being on SNPs within the OXTR. Using data from a large population-based dataset, no significant associations between SNPs in the OXTR and ASD were observed. However, the AG genotype from the rs53576 marker did provide evidence for a protective effect of OXTR on ASD (OR = 0.707, 95% CI: 0.512 – 0.975, p_{OR} = 0.0582) prior to adjusting for false discovery rate.

SNPs in the OXTR have previously been associated with social behaviour and ASD [4,7,8]. The four OXTR SNPs examined in this study were rs2254298, rs1042778, rs53576 and rs7632287. All of these SNPs have provided inconsistent results in previous studies done with smaller sample sizes [2-4]. In 2005, Wu et al. identified the transmission disequilibrium for the rs2254298A and rs53576A markers in 195 Chinese Han ASD trios [2]. Both of these SNPs are not involved in the functioning of the gene rather they are located in the third intron region of the OXTR [2,4,17]. It has been suggested that these two SNPs may work together with a third causal locus, such as the OXTR promoter region, to produce ASD [2]. A study by Jacob et al (2007) found a significant association between rs2254298G and ASD but did not report such an association within rs53576 in a study containing 57 Caucasian ASD trios [3]. In contrast to these two studies but in accordance with the Campbell et al. (2011) study, the current study did not find an association between either of these SNPs and ASD. Only one genotype from the rs53576
marker came close to demonstrating an association with ASD. Thus, it is possible that these two markers may be involved in the ASD phenotype (i.e. the severity of symptoms) without being directly involved in ASD status. For example, a study by Di Napoli et al. (2014) evaluated the association between these two SNPs, as well as seven others, and Asperger syndrome and found that neither the rs2254298A nor the rs53576A markers were significantly associated [18]. This is in contrast to their apparent association with ASD providing support for the idea that these markers are working in combination with a third locus that results in more severe social communication deficits.

The remaining two SNPs, rs1042778G and rs7632287G, were found to be significant in the 2011 study by Campbell et al. This study was the largest family-based sample to evaluate the relationship between OXTR SNPs and ASD, consisting of 1,238 pedigrees with a total of 2,333 individuals with ASD [4]. Despite the large sample size, after adjusting for multiple comparisons, none of the SNPs in this study were found to be significant. This is consistent with the results from the current investigation that found no statistically significant association between OXTR and ASD after adjusting for false discovery rate. Conflicting research has demonstrated that the rs1042778 marker is associated with inhibited sociality, aggression and ASD [19,20]. Another study did identify the rs7632287, not rs1042778, as being associated with pair-bonding traits, childhood social problems and social interaction deficit symptoms [21]. Furthermore, a meta-analysis by LoParo and Waldman (2015) found a strong relationship between rs7632287, ASD and social behaviour [22]. However, the authors acknowledged that studies to date have not had sufficient power to detect effect sizes suggesting that as the amount of genome-wide association studies increases so too will evidence supporting a relationship between OXTR SNPs and ASD [22].

The current study was not without limitations. First, the investigators were unable to stratify the results by ethnicity. Ethnicity plays an important role in the relationship between OXTR and ASD as shown by differences in risk alleles [2,3]. There were insufficient individuals with ethnicity data to allow
for comparisons to be made between ethnicities (i.e. some categories contained less than five people). As a result, potential associations that were implicated by previous research may have been masked due to genetic differences between the sibling-pairs of different ethnicities. Second, each SNP was evaluated separately to determine if there were any associations. It is unlikely that a single mutation within one chromosome is sufficient to result in the complete social communication ASD phenotype. Future research should evaluate the effect of haplotypes within these OXTR SNPs on ASD. In addition to examining haplotypes within these OXTR SNPs, a few additional SNPs in the CAV3 region should be evaluated as well. This is because the rs7632287 overlaps with the CAV3 region that is mediated by vasopressin, which is implicated in social behaviour and ASD [4,20,23]. Future research should also evaluate the effect of DNA methylation of the OXTR and ASD. Epigenetics has become a hot topic in the last 10 years, with DNA methylation status of the OXTR just recently being studied [24]. Gregory et al. (2009) demonstrated that individuals with ASD possess significantly increased DNA methylation within the peripheral blood cells and temporal cortex, and display a reduced expression of OXTR messenger RNA (mRNA) in their temporal cortex [24,25].

In conclusion, this study was one of the largest case-control studies to evaluate the relationship between OXTR polymorphisms and ASD. The results from the current study provided limited support of the involvement of OXTR with ASD demonstrating a weak negative association for the rs53576 marker after controlling for sex. This indicates that OXTR is a potential genetic variant that plays a role in the aetiology of ASD. However, ASD is likely the result of a combination of genetic risk factors and therefore, more research in this area is required.
4.6 References


Chapter 5

The severity of social communication deficits in verbal individuals with autism spectrum disorder resulting from genetic variation in the oxytocin receptor gene

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Amy McNaughton, PhD; Xudong Liu, PhD

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5.1 Abstract

The effects of four single nucleotide polymorphisms (SNPs) within the oxytocin receptor gene (OXTR) on resulting social communication deficits were examined in verbal children with autism spectrum disorder (ASD) from 235 simplex (SPX) families. A linear regression analysis demonstrated that the AG genotype from the rs2254298 marker was significantly associated with higher scores on the Autism Diagnostic Interview-Revised (ADI-R) compared to the GG genotype in both a mixed and Caucasian only sample. These results suggest that individuals with an AG genotype at this location have more severe social communication deficits. This SNP has previously been significantly associated with enlarged amygdalar volumes, which is implicated in the Theory of Mind (ToM) hypothesis of ASD. Future research is needed to identify the impact of these SNPs on brain function.
5.2 Introduction

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders characterized by impairments in two main symptom domains: (1) social interaction and communication, and (2) restrictive behaviour [1,2]. In 2013, the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) was released. The DSM-5 placed different subtypes of ASD on a spectrum based on different levels of severity rather than listing them as discrete conditions, and removed the criteria for a diagnosis to be made by age three [3,4].

The Autism Diagnostic Interview-Revised (ADI-R) is a standardized, investigator-based interview that seeks information related to the criteria required for a DSM-IV diagnosis of ASD [5-7]. It includes 93 items, scored between zero and three, which have been categorized into three functional domains: communication, reciprocal social interactions, and other behaviours (i.e. repetitive behaviours) [7-9]. More specifically, there are 41 questions on the ADI-R that deal with both verbal and non-verbal communication [7-9]. Appendix C provides the scoring scheme for the ADI-R social communication domain. While the ADI-R is based on the DSM-IV criteria, research has demonstrated that it is still capable of identifying the presence of symptoms in all required domains of the DSM-5 [10]. For example, Mazefsky et al. (2013) showed that 83% of their research participants diagnosed by the ADI-R still met the criteria for a DSM-5 diagnosis of ASD [10]. To estimate the severity of autistic symptoms, researchers have used overall scores or scores in a specific domain [9].

There is a wide range of variability in ASD and resulting symptom severity highlighting that there are no two cases that are the same [11]. This variability could be the result of the presence of different genetic and environmental factors. Specifically, genetic factors have been hypothesized to account for 70 percent of the variance (difference in phenotypes) seen in ASD [12,13]. Multiple genetic variants, such as those within FOXP2 and RELN genes, have been identified to play a role in ASD [14-
More recently, variants in the oxytocin receptor gene (OXTR) have been implicated for their potential effect on social communication within individuals with ASD [1,2,15,16]. The oxytocin neuropeptide is responsible for mediating social behaviours in humans and its receptor, encoded by the OXTR, is expressed within the amygdala [16]. The amygdala, which is a key area implicated in theory of mind (ToM; discussed further in Chapter 7), is believed to be responsible for allowing individuals to infer what oneself and others are feeling, and is involved in representing the mental states of both oneself and others [17,18].

Five different preliminary studies have examined the relationship between the OXTR and ASD. They have identified two different single nucleotide polymorphisms (SNPs) within the OXTR that are differently distributed among children with ASD: rs2254298 [1,2,19] and rs53576 [2]. Only a single study, by Campbell et al. (2011) has taken research one step further to examine the association of a wide range of OXTR variants with different phenotypic domains of ASD [11]. Although, they did not find the previous two SNPs to be significantly associated with ASD, they showed that rs2268493T, rs1042778G, and rs7632287G [19] SNPs are significantly associated not only with case status but also with categorical phenotypic cutoff scores on the ADI-R [11]. Campbell et al. (2011) did not find significant results after correcting for multiple comparisons, and therefore further research needs to be done to evaluate this relationship. In 2010, Tansey et al. identified rs7632287A as being significantly associated with an increased risk of ASD [20]. This marker overlaps with the CAV3 region that is mediated by another social neuropeptide, vasopressin [11,21,22]. The purpose of this paper is to evaluate and describe the association between the twelve OXTR genotypes, provide from four SNPs (rs2254298, rs7632287, rs1042778 and rs53576), and social communication deficits among individuals with a diagnosis of ASD.
5.3 Methods

5.3.1 Participants

Participants for the study were selected from the Autism Spectrum Disorders – Canadian-American Research Consortium (ASD-CARC; www.autismresearch.ca) registry. A diagnosis of ASD was based on the DSM-IV criteria [8]. Participants were eligible for this study if they had a diagnosis (by clinician) of ASD in the absence of a known aetiology (i.e. excluding karyotype-detectable chromosomal anomalies and Fragile X syndrome) or other neurodevelopmental disabilities (i.e. Down syndrome), had a score on the ADI-R, were from a simplex (SPX) family (only family member with a diagnosis of ASD), were verbal and were older than three at the time of assessment. There were 275 participants who met these criteria. Ethics approval for this study was obtained from the Queen’s University Health Sciences Research Ethics Board.

5.3.2 Materials

Participants were genotyped using custom, validated TaqMan SNP Genotyping Assays (http://www.lifetechnologies.com) on an ABI Prism ViiA7 Real-Time PCR machine in 384-well plates. The Sequence Detection System (SDS) ViiA7 (Version 1.2) software with standard parameters was used to automatically score the genotypes. Accuracy of the genotyping procedure was evaluated using duplicated samples and negative controls in each plate. The four SNPs genotyped were rs2254298, rs7632287, rs1042778, and rs53576.

ADI-R information was provided from the ASD-CARC database for the participants included in the study. The social communication scores were the focus of this study. This domain evaluates both verbal and non-verbal communication. For example, it asks whether or not there is a delay or total lack of language not compensated by gesture, if there is relative failure to initiate or sustain conversational interchange, if there is stereotyped and repetitive use of language, etc. [7-9]. However, the diagnostic
algorithms for verbal and non-verbal participants are different; the ADI-R social communication score is typically dichotomized using a standard cutoff of greater than or equal to eight for verbal subjects and greater than or equal to seven for non-verbal subjects to meet the minimum score for this domain according to the DSM-IV [9]. Thus, it was decided a priori to evaluate verbal and non-verbal subjects separately. An individual is classified as being verbal or non-verbal based on item 30 on the ADI-R [23]. This question examines the overall level of language in the individuals being tested, including the number of words or phrases used, if they use verbs, and if they are understood [23]. A non-verbal designation is given if the individual has a score of one or two on this item [23]. This manuscript only includes results for verbal subjects (N = 235). Chapter 6 presents the results obtained from non-verbal individuals (N = 40). This study treated the social communication score as a continuous variable to examine the association between the degree of social communication deficit and the twelve genotypes produced by the four SNPs in the OXTR.

5.3.3 Statistical Analysis

The relationship between the four SNPs (rs2254298, rs7632287, rs1042778, and rs53576) and resulting social communication deficits was evaluated using linear regression with an additive genetic model approach. The association between four variables (sex, age at ADI-R assessment, interviewee relationship, and interview format) and social communication scores were evaluate using ANOVA. These variables were investigated as potential confounders using a change-in-estimate criterion of ten percent. The false discovery rate of the four SNPs was controlled for using the Benjamini-Hochberg adjustment.

5.4 Results

A total of 235 verbal participants were selected for this study. Eighty-four percent of the sample was male. Eighty-eight percent of the sample was Caucasian. The mean age of participants was 22.5 (14 – 51) years, with the average age at ADI-R administration being 11.5 (9 – 19) years old. Approximately 90
percent of the participants were assessed using the 2003 version of the ADI-R, with the majority being interviewed over the phone (78%) and the interviewee being the participants’ mother (90%).

*Tables 5.1 and 5.2* summarize the allele and genotype frequencies in all individuals with ASD, within the study (N = 235) and when the sample was restricted to Caucasians only (n = 188) for the rs2254298, rs53576, rs7632287, and rs1042778 markers respectively. *Table 5.3* present the means and associated standard deviations for ADI-R social communication scores based on the participants’ sex, age at ADI-R assessment, interviewee relationship, interview format, and genotypes.

As shown in *Table 5.4*, the AG genotype provided by rs2254298 marker was found to be significantly associated with higher social communication scores on the ADI-R in verbal participants after accounting for sex, age at ADI-R assessment, interviewee relationship (mother, father, both), and interview format (telephone, face-to-face) (n = 187, Parameter estimate = 1.833, SE = 0.762, p = 0.0171). When restricted to only Caucasian participants, a significant relationship was seen after accounting for sex, interview format, and interviewee relationship (n = 159, Parameter estimate = 2.20, SE = 0.838, p = 0.0094). The AA marker within this SNP was not found to be associated with increased social communication deficits prior to or following a restriction to Caucasians. No associations were found when the relationship was evaluated in non-verbal subjects (refer to *Table 6.3* in Chapter 6).

**5.5 Discussion**

The AG genotype from rs2254298 was the only marker to demonstrate a significant association, specifically with higher social communication scores on the ADI-R prior to correcting for false discovery rate (see *Table 5.4*). This implies that the AG genotype leads to higher social communication scores on the ADI-R indicating a more severe symptomology (more deficits within the social interaction and communication domain) compared to a GG genotype. This aligns with previous research demonstrating that the ‘A’ allele, predisposes individuals to different psychiatric disorders and psychopathy [25], and
that this polymorphic variation (AG genotype) is associated with greater social anxiety, low sociability [25,26] and greater amygdalar volumes [25,27,28]. Thus, it is plausible that the AG genotype provided by the rs2254298 marker results in greater volume size of the amygdala leading to an increased risk of psychopathological syndromes, such as ASD, and greater deficits in ToM processing. Furthermore, the results from this study suggest that this genotype may contribute to a more severe symptomology associated with ASD (i.e. autism rather than Asperger’s disorder as described in the Diagnostic and Statistical Manual of Mental Disorders [DSM], Fourth Edition – Text Revision) that may be due to the increase amygdalar volumes previously described.

While this may be the case, further research has looked solely at the effect of OXTR SNPs in individuals and ASD [1,2,11,19,25,29]. These studies have demonstrated that the ‘A’ allele from the rs2254298 marker is the risk allele in Chinese [1] and Japanese [25,29] samples whereas the ‘G’ allele has been denoted as the risk allele in Caucasians [2] and Israeli [19,23] samples. Researchers have attributed this difference to population genetics that suggests different ethnicities express the ‘A’ allele at different frequencies [25]. They explain that the ‘A’ allele is more common in Asian populations, with only 40 to 50 percent exhibiting the GG genotype that is very common in European populations where the ‘A’ allele is uncommon [25,30]. It is surprising then that the results (see Table 5.4) from this study found a relationship between the AG genotype and higher social communication scores, indicating more severe social communication deficits in individuals with ASD, when restricting the sample to Caucasians only while keeping the comparison genotype as GG. This suggests that the ‘A’ allele may be the risk allele, regardless of ethnicity, and that previous research had too small of a sample size to be able to evaluate the effect of ‘A’ in Caucasian populations. This is supported by the fact that the AA genotype is usually too rare in these studies and therefore, typically removed from analysis [2,25,26].
The clinical significance of this result remains unclear as the majority of the participants within each genotype were above the cut-off score for the social communication domain of the ADI-R. Specifically, 81.3 percent of individuals with the AG genotype were above the ADI-R cut-off score compared to 81.8 percent and 78.4 percent of individuals with AA and GG genotypes respectively. Furthermore, the mean ADI-R social communication scores for verbal participants with the AG genotype were 19.18 compared to 18.78 and 17.33 scores for the AA and GG genotypes respectively (see Table 5.3). Each of these scores is well above the cut-off score of eight for this domain in verbal individuals indicating that they meet the criteria for deficits within the domain [9]. Thus, the AG genotype at the rs2254298 may not play a role in the severity of symptoms but rather may be important in the development of impairments within the social interaction and communication domain due to its functional effect on the amygdala.

Two limitations should be considered when interpreting the results of the current study. First, this study was unable to stratify the analyses by ethnicity due to too small of a sample size within some of the categories. Ethnicity has been demonstrated to play a significant role in the relationship between OXTR and ASD phenotypes due to population genetics [25]. As a result of population genetics, different alleles are risk-inducing depending on the ethnicity of the individual providing the genetic sample [25]. This is seen in the rs2254298 marker that has different risk-inducing alleles depending on whether the individual is Chinese [1] or Caucasian [2]. Thus, it is possible that potential associations within the markers that provided insignificant results may have been masked because of the presence of multiple different ethnicities in the sample. However, when restricting our sample to Caucasians only, the relationships between these markers and ASD phenotypes remained insignificant. This implies that ethnicity may not play as big of a role in the relationship between OXTR and social communication scores as originally believed. Second, each SNP was evaluated separately to determine if there were any associations rather
than evaluating them as haplotypes. It is unlikely that a single mutation within one chromosome is sufficient to result in the impairments seen in the social interaction and communication domain. Future research should evaluate the effect of haplotypes within the OXTR and ASD phenotypes. In addition to examining haplotypes within these OXTR SNPs, a few SNPs in the CAV3 region should be evaluated as well. This is because the rs7632287 overlaps with the CAV3 region that is mediated by vasopressin, which is implicated in social behaviour and ASD symptomatology [14,21,22].

In conclusion, this study evaluated the relationship between OXTR SNPs and social communication deficits. It found an association between the AG genotype in the rs2254298 with higher social communication deficits. While significant, this result has provided contrary results to previous research in Caucasian populations. Thus, future research is needed to further evaluate this relationship. Future research should also focus on the function of specific OXTR SNPs within the brain.
5.6 References


Table 5.1 Allele and genotype distributions of OXTR rs2254298, rs53576 and rs7632287 among verbal children with ASD in the complete (mixed) sample and a restricted sample of Caucasians

<table>
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<tr>
<th>Marker</th>
<th>Allele</th>
<th>Genotype</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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</tr>
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<td></td>
</tr>
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<td>Caucasians</td>
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</table>

*the numbers in brackets represent percentages

Table 5.2 Allele and genotype distribution of OXTR rs1042778 among verbal children with ASD in the complete (mixed) sample and a restricted of Caucasians in verbal children with ASD

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<th>HWE</th>
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*the numbers in brackets represent percentages
Table 5.3 Mean ADI-R social communication scores among verbal children with ASD, and associated standard deviation, for sex, interviewee relationship, interview type, age at ADI-R administration, and genotypes (N = 235)

<table>
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<tr>
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<th>ADI-R Social Communication Score</th>
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</thead>
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<tr>
<td></td>
<td>Mean</td>
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<tr>
<td>Name Levels</td>
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<td>Female</td>
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<tr>
<td>Interviewee Relationship</td>
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<tr>
<td>Other</td>
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<tr>
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<tr>
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<td>GG*</td>
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*indicates the reference group. Other group for interviewee relationship is comprised of fathers or both parents.
Table 5.4 Parameter estimates for the associations between OXTR SNPs and social communication scores on the ADI-R among verbal children with ASD in complete (mixed) sample and in the Caucasian only sample after accounting for sex, interview type and interviewee relationship

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<td>0.9464</td>
<td></td>
</tr>
</tbody>
</table>

a indicates unadjusted p-value
b indicates FDR-adjusted p-value
Chapter 6
Additional Results

This chapter contains the results of non-verbal participants and two sensitivity analyses done for objective two.

6.1 Non-Verbal Participants

Individuals within the study that were given a score of one or two on item 30 of the Autism Diagnostic Interview-Revised (ADI-R) were classified as non-verbal [1]. A total of 40 participants were eligible for the study. Only a single study, by Campbell et al. (2011), has examined the relationship between oxytocin receptor gene (OXTR) single nucleotide polymorphisms (SNPs) and autism spectrum disorder (ASD) in non-verbal individuals [2]. Earlier studies were unable to examine this relationship due to small sample sizes [3-5]. Table 6.1 and 6.2 summarize the allele and genotype frequencies for all non-verbal individuals in the study (N = 40). No associations were found between these four OXTR SNPs and social communication deficits (Table 6.3). These findings are consistent with Campbell et al. (2011) [2]. However, it is possible that different OXTR SNPs are involved in the social communication deficits displayed by non-verbal individuals. Future research is needed to evaluate this relationship in individuals who are non-verbal.

6.2 Sensitivity Analyses

Two variables, interviewee relationship (mother, father, both) and interview format (telephone, face-to-face) were significantly associated with social communication scores on the ADI-R (see Table 5.5 in Chapter 5). Due to smaller sample sizes for these analyses the AG and AA genotypes were grouped together for the rs2254298, rs53576 and rs7633287 markers, and the GT and TT genotypes were grouped together for the rs1042778 marker. The reference group was GG for all markers. In addition, the analyses
were only conducted in verbal participants because the sample of non-verbal was comprised of less than 20 children.

6.2.1 Mothers Only Sample

Typically, only a single informant, normally the mother, is used to gather information about a child’s autistic traits with information from the father or teachers missing [6-8]. This represents a potential issue because mothers, fathers and teachers, due to unique personal experiences or situational specificity, may interpret certain behaviours exhibited by the child differently [6]. This thesis demonstrated that there was a significant difference in the ADI-R social communication domain scores between those ADI-R interviews completed with the mother versus another interviewee (p = 0.0465). The relationship between OXTR SNPs and resulting social communication scores was evaluated in a sample that contained only mothers (90% of the original sample). Table 6.4 provides the results of a linear regression evaluating this relationship. The rs2254298A marker was found to be significantly associated with higher social communication scores on the ADI-R after accounting for the sex of the child and interview type (telephone versus face-to-face) (n = 172, Parameter estimate = 1.6388, SE = 0.720, p = 0.0241) prior to adjusting for false discovery rate. This finding reflects what was seen in Chapter 5 but is not directly comparable as the AA and AG genotypes were combined for this analysis. This suggests that the type of respondent is not likely affecting the relationship between the four OXTR SNPs and social communication scores.

6.2.2 Telephone Only Sample

The ADI-R is the ‘gold standard’ originally used for a clinical diagnosis of ASD [9]. It is a semi-structured interview that is completed face-to-face [9]. However, the administration of this interview has started to occur over the telephone as researchers have begun using the ADI-R scores for their studies [9]. In 2010, Ward-King et al. showed that there was no significant difference in the scores obtained on the
ADI-R when the interview was conducted over the phone or in person stating that the scores obtained from either mode are reliable [9]. In contrast, this thesis demonstrated that there was a significant difference in social communication scores on the ADI-R depending on the mode of interview (telephone/face-to-face) in the complete sample ($p = 0.0189$) used in Chapter 5. The relationship between OXTR SNPs and resulting social communication scores was evaluated in a sample of participants that were interviewed over the phone (78% of the original sample). Table 6.5 provides the results from this analysis. No associations were seen after restricting the sample to those who completed the interview over the phone. This suggests that the observed difference in the rs2254298 marker could be due to the inclusion of social communication data obtained from different protocols (telephone or face-to-face).

6.2.3 Conclusion

Based on these sensitivity analyses, the results from the full model may not hold because of the inclusion of individuals whose ADI-R interviews were done over the telephone compared to those done face-to-face.
Table 6.1 Allele and genotype distributions of OXTR rs2254298, rs53576 and rs7632287 among non-verbal children with ASD in the complete (mixed) sample and a restricted sample of Caucasians

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele</th>
<th>Genotype</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;sup&gt;c&lt;/sup&gt; (df = 1)</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>rs2254298</td>
<td>Mixed</td>
<td>78</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>(84.6)</td>
<td>(15.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caucasians</td>
<td>58</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>(82.8)</td>
<td>(17.2)</td>
<td></td>
</tr>
<tr>
<td>rs53576</td>
<td>Mixed</td>
<td>78</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>(68.0)</td>
<td>(32.0)</td>
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</tr>
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<td></td>
<td>Caucasians</td>
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</tr>
<tr>
<td></td>
<td>(67.2)</td>
<td>(32.8)</td>
<td></td>
</tr>
<tr>
<td>rs7632287</td>
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<td>55</td>
</tr>
<tr>
<td></td>
<td>(72.4)</td>
<td>(27.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caucasians</td>
<td>56</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>(71.4)</td>
<td>(28.6)</td>
<td></td>
</tr>
</tbody>
</table>

*the numbers in brackets represent percentages

Table 6.2 Allele and genotype distributions of OXTR rs2254298, rs53576 and rs7632287 among non-verbal children with ASD in the complete (mixed) sample and a restricted sample of Caucasians

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele</th>
<th>Genotype</th>
<th>HWE</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;sup&gt;c&lt;/sup&gt; (df = 1)</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>G</td>
<td>T</td>
</tr>
<tr>
<td>rs1042778</td>
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<td>41</td>
</tr>
<tr>
<td></td>
<td>(52.6)</td>
<td>(47.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caucasians</td>
<td>58</td>
<td>30</td>
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<tr>
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<td>(51.7)</td>
<td>(48.3)</td>
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*the numbers in brackets represent percentages
Table 6.3 Parameter estimates for the associations between OXTR SNPs and social communication scores on the ADI-R among non-verbal children with ASD in complete (mixed) sample and in the Caucasian only sample after accounting for sex, interview type and interviewee relationship

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Mixed Sample</th>
<th></th>
<th></th>
<th>Caucasians Only</th>
<th></th>
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</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Genotype</td>
<td>Parameter Estimate</td>
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<td>p&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>OXTR</td>
<td>rs2254298</td>
<td>39</td>
<td>AA</td>
<td>0.3507</td>
<td>0.8025</td>
<td>0.8962</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AG</td>
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</tr>
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<td>0.7751</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AG</td>
<td>0.0926</td>
<td>0.8962</td>
<td>0.8962</td>
<td></td>
</tr>
<tr>
<td>OXTR/CAV3</td>
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<td>39</td>
<td>TT</td>
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<td>0.4055</td>
<td>0.7101</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GT</td>
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<td>0.0789</td>
<td>0.6312</td>
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<td>OXTR</td>
<td>rs53576</td>
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<td>AA</td>
<td>0.9186</td>
<td>0.4004</td>
<td>0.7101</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AG</td>
<td>0.5233</td>
<td>0.4438</td>
<td>0.7101</td>
<td></td>
</tr>
</tbody>
</table>

a indicates unadjusted p-value
b indicates FDR-adjusted p-value

Table 6.4 Parameter estimates for the associations between OXTR SNPs and social communication scores on the ADI-R, completed by mothers only, among verbal children with ASD in complete (mixed) sample and in the Caucasian only sample after accounting for sex and interview type

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
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<th></th>
<th></th>
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<th></th>
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<tr>
<td></td>
<td></td>
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<td>Genotype</td>
<td>Parameter Estimate</td>
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<td>p&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N</td>
</tr>
<tr>
<td>OXTR</td>
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<td>0.0241</td>
<td>0.0964</td>
<td>147</td>
</tr>
<tr>
<td>OXTR/CAV3</td>
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<td>rs53576</td>
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<td>AA/AG</td>
<td>-0.5454</td>
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<td>169</td>
<td>AA/AG</td>
<td>-0.9700</td>
<td>0.1273</td>
<td>0.2546</td>
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</table>

a indicates unadjusted p-value
b indicates FDR-adjusted p-value
Table 6.5 Parameter estimates for the associations between OXTR SNPs and social communication scores on the ADI-R, completed by telephone, among verbal children with ASD in complete (mixed) sample and in the Caucasian only sample after accounting for sex and interviewee relationship

<table>
<thead>
<tr>
<th>Gene</th>
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</tr>
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<td>0.9753</td>
<td>147</td>
<td>AA/AG</td>
</tr>
<tr>
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<td>-0.8876</td>
<td>0.1733</td>
<td>0.3466</td>
<td>144</td>
<td>TT/TG</td>
</tr>
</tbody>
</table>

a indicates unadjusted p-value

b indicates FDR-adjusted p-value
6.3 References


Chapter 7

Overall Discussion

The overall purpose of this thesis was to contribute to existing knowledge surrounding the aetiology of ASD. Specifically, it looked at the effects of four different single nucleotide polymorphisms (SNPs) within the oxytocin receptor gene (OXTR) on both ASD risk and resulting severity of social communication deficits in individuals diagnosed with ASD. Data was obtained from the Autism Spectrum Disorders – Canadian-American Research Consortium (ASD-CARC) resulting in one of the largest population-based samples to evaluate this relationship. Conditional logistic and linear regressions were used to evaluate the data from objective one and two respectively. This chapter will outline the main findings from the two objectives, methodological strengths and limitations, the contribution of these results to current research, and where future research should continue in this area.

7.1 Summary of Main Findings

While the literature identified five OXTR SNPs of interest, only four were included due to an observed rarity of the rs226893 marker in a single 382-well plate (refer to Figure 3.2 in Chapter 3).

7.1.1 SNPs of OXTR and ASD

The first objective, as described in Chapter 4 of this thesis, compared the genotype distributions of four OXTR variants (rs2254298, rs7632287, rs1042778 and rs53576) in individuals with ASD to that in their unaffected siblings. The study consisted of 607 sibling pairs (N_{total} = 1214 participants) where one of the pairs had been previously diagnosed (by a clinician) with ASD. Sex was the only variable that was found to confound the relationship between OXTR SNPs and ASD when examined using a change-in-estimate approach and backwards selection to test for potential confounders. This was expected due to the sex ratio seen in ASD.
Rs53576 was the only OXTR SNP implicated in ASD status. This polymorphism is the result of a change in the genotype from a G to an A allele within the intron area of the OXTR on chromosome 3, which contains the oxytocin receptor gene [1]. Individuals with a GG (wild type) genotype at this marker have been associated with higher sociality than the two other possible genotypes (AG/AA) [2]. Specifically, these individuals (GG) are empathetic [3], have higher social auditory ability [4], and display greater general sociality as rated by peers [5]; whereas, individuals with the AG and AA genotypes display deficits in these areas [2-5]. The last two genotypes have also been implicated in several social related psychiatric disorders, including ASD [2,6]. Only a single genotype (AG), within this thesis, appeared to be significantly associated with case status prior to adjusting for multiple comparisons [OR = 0.707; 95% CI: 0.512 – 0.975]. This indicates that the AG genotype may have protective effect; however, this finding was not seen after adjusting for multiple comparisons (see Table 4.3 in Chapter 4). This is inconsistent with literature within the area which has either found no association [7-9] or a harmful effect [6] resulting from the presence of the A allele. Ethnicity does represent a potential explanation for these inconsistent effects. In 2005, Wu et al. found that rs53576A was significantly associated with autism in a Chinese population [6]. In contrast, this marker was not significantly associated with autism in three different Caucasian only samples [7-9]. No association was seen, prior to or after adjusting for false discovery rate, between this marker and ASD status when the sample was restricted to Caucasians only.

It is surprising that none of the SNPs studied were significantly associated with case status even following the restriction to a solely Caucasian sample. This is because previous research in this area has found each of these SNPs to be significant when evaluated in smaller sample sizes in specific ethnicities (Caucasians only, Asians only, etc.) [6,7,9,10]. Specifically, a study using 57 Caucasian trios found rs2254298A to be significantly associated (p = 0.03) with ASD [7]. Rs7632287G was demonstrated as
being significantly associated with case status in an Irish sample only (N = 177) and in a combined sample from Ireland, Portugal and the United Kingdom (N = 436) [10]. In comparison, this study evaluated the relationship between OXTR and ASD in multiple ethnicities together. Our sample was comprised of 304 Caucasian sibling-pairs, seven Asian sibling-pairs, four Southeast Asian sibling-pairs, three African-American sibling-pairs, three First Nations sibling-pairs, and 27 ‘Other’ sibling-pairs (N = 1214; n_case = 607). Due to the cell sizes for each ethnicity, the analyses could not be stratified to look at the effect within specific ethnicities. It is possible that any relationship was masked due to the inclusion of multiple ethnicities; however, ethnicity (Caucasian vs. Other) did not represent a potential confounder when evaluated. Furthermore, when restricting the sample to only Caucasians, no associations were found between any of the SNPs and ASD.

However, two issues with the data could explain these null findings. First, low power has previously been and remained an issue. In a study by Campbell et al. (2011) power was an issue when these SNPs, along with 21 others, were evaluated in the largest study sample (N = 5,432; n_case = 2,333) known to the investigators [8]. They found that the rs2268493T (p = 0.043), rs1042778G (p = 0.037) and rs7632287G (p = 0.016) were significantly associated with ASD but these relationships did not survive after correcting for multiple comparisons [8]. They suggest that these relationships still contribute to ASD risk because these results provide “additional suggestive evidence in support of previous genetic association data from smaller, independent family cohort, as well as biological plausibility (p.107)” [8]. In addition, they do not rule out the possibility of other SNPs, such as rs53576 and rs2254298, being involved in an increased risk of ASD. In an attempt to resolve the power issues from the Campbell et al. (2011) study, the current investigation evaluated fewer SNPs (N = 4) than previous genetic association studies. This did not largely improve power in the current investigation. Future research should focus on improving the power in an attempt to determine the relationship between these SNPs and ASD.
Finally, the genotypic and allelic frequencies for the SNPs among the cases in our study (see Table 4.1 and 4.2 in Chapter 4 under results) are not consistent with previous research. For example, the rs2254298 marker, the overall genotypic and allelic frequencies for the affected individuals were 434 (73.0%) GG, 137 (23.0%) AG and 24 (4.0%) AA, and 1005 (84.4%) G and 185 (15.6%) A respectively. In a sample of Caucasians only and a separate sample of Chinese individuals only, different genotypic and allelic frequencies were observed for the same marker [6,7]. In the Caucasian sample specifically, the genotypic and allelic frequencies for affected individuals were 47 (82.5%) GG, 10 (17.5%) AG and 0 (0.0%) AA, and 104 (91.2%) G and 10 (8.8%) A respectively [7]. When restricting our sample to Caucasians only, the genotypic and allelic frequencies were 227 (74.3%) GG, 63 (20.7%) AG and 15 (4.9%) AA, and 517 (84.7%) G and 93 (15.3%) A respectively in affected individuals. However, the frequencies in non-affected individuals within this study were consistent with those seen in previous research [7]. These two issues must need to be addressed by future research prior to concluding that these SNPs do not play a role in ASD status.

7.1.2 SNPs of OXTR and Severity of Social Communication Deficits

Objective two, as described in Chapter 5 of this thesis, evaluated the association of four SNPs (rs2254298, rs7632287, rs1042778 and rs53576) and the resulting social communication deficits among individuals diagnosed with ASD. A sample of 235 individuals (M: 22.5 ± 5.9 years old) was used to examine the relationship between the OXTR and social communication deficits. The AG genotype from rs2254298 was significantly associated with higher social communication scores in verbal participants on the Autism Diagnostic Interview-Revised (ADI-R), prior to adjusting for multiple comparisons, indicating more severe deficits in this domain (see Table 5.4 in Chapter 5). This genotype demonstrated a similar finding in the verbal participants from a Caucasian only sample (n = 187). The importance of this finding is described below in section 7.4. No associations were found among any genotype and social
communication deficits in non-verbal participants in either the complete sample or the Caucasian only sample (see Table 6.3 in Chapter 6). The results of the sensitivity analyses (see Tables 6.4 and 6.5 in Chapter 6) call into question the validity of the results presented in Chapter 5. Sex was the only variable that was found to confound the relationship between the OXTR SNPs and ASD when examined using a change-in-estimate approach and backwards selection to test for potential confounders. This was expected due to the sex ratio seen in ASD.

The remaining markers (rs1042778, rs53576, and rs7632287) were not significantly associated with social communication scores on the ADI-R. Previous research in the area had found a significant association between these markers and ASD [6-8]. The rs1042778G marker was implicated by a single study, Campbell et al. (2011), where it was shown to be associated with ASD [8]. After adjusting for multiple comparisons, this marker was no longer significantly associated with ASD, similar to this investigation, and therefore may not be directly involved in the relationship. Rs53576A was found to be significantly associated with ASD in a small sample of Chinese trios, but this finding was not replicated in a Caucasian sample [6,7]. The findings in Caucasian populations are consistent with the current investigation that did not find this marker to be associated with social communication deficits (Table 5.4 in Chapter 5). Finally, the ‘A’ allele from the rs7632287 marker was originally believed to risk inducing. For example, two previous studies found the rs76322987A marker to be significantly associated with ASD [11] and social interaction issues [12]. In addition, previous research has found the rs7632287G marker to be associated with deficits in overall socialization, such as social communication and social cognition [13]. However, Campbell et al. (2011) provided evidence to the contrary when they demonstrated a significant association between the rs7632287G marker and the social communication domain of the ADI-R in a predominately Caucasian sample [8]. The current investigation did not find a significant association but did implicate the rs7632287A allele as being potentially associated with lower
social communication scores on the ADI-R in verbal participants (p_\text{Caucasians} = 0.0884). This indicates that the ‘A’ allele may be associated with fewer deficits within this domain. Thus, more research focusing on this markers relationship with ASD risk and phenotypes is needed.

**7.2 Strengths and Limitations**

Research into this area is limited. Only five studies investigating these SNPs with respect to ASD risk and ASD phenotypes have been published to date. Each of these studies, except one, was done with small samples in specific ethnicities, without distinguishing ASD phenotypes. Using the data from the ASD-CARC, the sample size for this thesis was larger than what has been used in four of these previous studies. While a study by Campbell et al. (2011), did evaluate 25 different SNPs in the OXTR in a large family-based sample, their study suffered from multiple comparisons that may have led to a higher probability of false positives [8]. This thesis restricted the analysis to four specific SNPs, identified from these previous studies, to reduce the probability of false positive results.

Potential misclassification errors, both for the exposure and outcome, were limited in this thesis due to the nature of the measurement tools. The TaqMan SNP Genotyping procedure, which was used to measure the exposure, is a common and thoroughly described protocol that uses a specific assay based on the SNP being evaluated [14]. This protocol is used to produce genotypes with high quality control [14]. As a result, false positives resulting from inconsistent DNA preparation and genotyping methods between plates, and SNPs leading to differential biases [15] and low genotype call rates [16] would have been limited. This is important as the inclusion of low quality DNA samples and imperfect genotyping assays would result in an increase in non-differential misclassification [16]. This study is based on a valid and reliable measure of the outcome variable: social communication. The second objective of this study aimed to determine if SNPs in the OXTR are associated with different social communication scores on the ADI-R. The ADI-R is a validated measure that is administered by trained clinicians to caregivers [17,18]. It has
high inter-rater ($\kappa = 0.62$ to $0.93$) and test-retest reliability ($\kappa = 0.93$ to $0.97$) [37,38]. This measure has also been found to be internally valid [17,18]. In addition, this measure is based on criteria from both the DSM-IV and the ICD-10 [17,18].

This thesis is not without limitations. First, while misclassification errors were reduced due to the nature of the exposure and outcome measurements, there is a chance that siblings who were originally classified as being unaffected may have been yet undetected cases of ASD. As such, these individuals would have been classified as controls in manuscript one resulting in misclassification of the outcome. To reduce this misclassification, controls that were older than the age of three were used since it is by this time that a diagnosis of ASD typically occurs, particularly in families where one child is already diagnosed.

Second, even though the ADI-R is a valid measure, it is also based on the DSM-IV diagnostic criteria rather than the current DSM-5 criteria. It was decided to still use this measure for the outcome because it has been demonstrated that the ADI-R is still capable of identifying the presence of symptoms in all required domains of the DSM-5 [19]. For example, Mazefsky et al. (2013) showed that 83 percent of their research participants diagnosed by the ADI-R still met the criteria for a DSM-5 diagnosis of ASD [19]. Furthermore, this measure is typically administered at age two for clinical reasons [20-22], however, a research ADI-R could be administered at any time after the age four. The average age of ADI-R administration in the sample for manuscript two was 11.5 years (range: 9 – 19). Since this tool is used to measure early behavioural signs that are characteristic of ASD [20-22], recall error may be present the older a child is because the interviewee is asked about behaviours that their child displayed at the age of two. The interviewee may not remember if their child displayed these behaviours or how severe they experienced these deficits. As a result, the social communication scores obtained from an ADI-R administered for research purposes may be lower than those obtained from an ADI-R administered by a
clinician at the time of diagnosis. For example, a study by Soke et al. (2011) found that scores in the social interaction and communication domain decreased significantly when an individual was assessed at age four compared to when they were assessed just two years earlier [22]. Thus, it is possible that these SNPs were not found to be significantly associated with higher social communication scores in the current sample due to recall error.

Third, while it has become clear that the aetiology of ASD is multifactorial, likely being the result of GxE interactions, this study focused only on a genetic aspect in its simplest form: single OXTR SNPs. This study is limited in that it did not evaluate haplotype formation between the four SNPs of interest. Post-hoc analysis of linkage disequilibrium was carried out (see Appendix D). In addition, it did not evaluate GxE interactions due to the complexity involved, and related resource and time requirements. Another reason for avoiding GxE interaction studies is that there is not enough information known about the different genetic or environmental factors that are working together to contribute to ASD. Thus, this thesis has provided limited support for the role of the OXTR in ASD risk and symptoms severity. This provides a foundational brick upon which future research considering environmental factors, such as maternal stress during pregnancy or obstetric complications, can be explored further. Ultimately, this will lead to a greater understanding of the different factors contributing to ASD aetiology.

Finally, and most importantly, this thesis was unable to stratify by ethnicity in either manuscript. This is a limitation as previous research has implicated different alleles as being risk-inducing based on ethnicity. For example, in a Chinese population the rs2254298A was demonstrated as being risk-inducing [7] whereas the rs2254298G was identified as being risk-inducing in a Caucasian population [8]. This difference is accounted for by population genetics, which finds that different alleles are more likely to be passed from one generation to another and that these differ depending on ethnicities [23]. However, as
mentioned in Chapter 5 and above, the inability to stratify by ethnicity may not be a major limitation, as the results remained the same when restricting to Caucasians only.

Post-hoc calculations revealed that the study’s power was low. Power refers to the ability to detect a true effect. It is common for genetic studies to lack power [40] due to a large amount of multiple comparisons when investigating a large number of SNPs [39,40] however, having selected a limited number of SNPs based on available empirical evidence, correction for multiple comparisons is not essential. Each of the five previous studies in the area had issues with power. This thesis attempted to improve on this issue by evaluating fewer SNPs and using a larger sample size. Another method for improving the power associated with different studies is to ensure that the disease model (additive, dominant, or recessive) is also accounted for within the power calculation [39]. For example, Hong and Park (2012) demonstrated that a smaller sample size is needed when using a dominant model and/or studying a common disease. Another possible way to improve the power is to include more controls than cases (i.e. 4 controls to 1 case) [39,40]. While larger samples are unlikely to be available to prove improved power, meta-analyses of the results from multiple studies, including this one, show promise.

7.3 Generalizability

To be eligible for manuscript one, participants needed to have a confirmed diagnosis (by clinician) of ASD without a known aetiology (i.e. excluding karyotyping-detectable chromosomal anomalies and Fragile X syndrome), from a simplex (SPX) family (only family member with a diagnosis of ASD), did not have other neurodevelopmental disabilities (i.e. Down syndrome), were older than the age of three, and have an unaffected sibling in the registry. Criteria for manuscript two included the same as outlined in manuscript one, however the participants also had to have an ADI-R social communication score. The results from these studies are unlikely to be generalized to individuals diagnosed with non-idiopathic ASD or from multiplex families as different genetic variants are likely to play a role in ASD risk. In
addition, ethnicity of the individual is likely to play a role in this relationship. Thus, these results may only be generalized to Caucasian individuals. However, the relationships were similar in the complete sample with mixed ethnicities and in the Caucasian only sample implying that these results may be consistent when evaluated in single ethnicity samples around the world.

7.4 Contributions of Research

The purpose of this thesis was to evaluate the relationship between OXTR SNPs, and ASD and social communication deficits. While no association was observed after adjusting for multiple comparisons for overall ASD risk, manuscript two is novel in that it is only the second study, to the knowledge of the investigators, to examine these SNPs with respect to a specific phenotype (i.e. social communication deficits). The first study to evaluate the relationship was Campbell et al. (2011) [8]. The current results suggest that there is a significant association, prior to adjusting for multiple comparisons, between the AG genotype produced by the rs2254298 marker and higher social communication deficits in individuals with ASD. As participants are continuously enrolling with the ASD-CARC, in time this association could be examined with greater power in different ethnicities.

The biggest finding in this thesis was the relationship between the AG genotype in the rs2254298 marker and higher social communication scores on the ADI-R that was significant prior to adjusting for false discovery rate. This is important because this SNP has previously been associated with larger amygdalar volumes in human subjects [23-25]. A larger amygdalar volume has been implicated in the theory of mind (ToM) hypothesis of ASD described by Baron-Cohen in 2001 [26]. ToM dictates that humans are able to infer what others are feeling, how they themselves are feeling, and how both will react in certain situations [26-28]. Specifically, researchers believe that individuals with ASD do not completely acquire false belief understanding, which is a key social-cognitive milestone, where the child is able to view mental states as independent ‘subjective representations’ of the world rather than being
concrete [28]. These impairments serve as a potential explanation for the communication and reciprocal social interaction failures that are demonstrated by individuals with ASD, as well as individuals afflicted with other language impairments [28,29]. Two areas of the brain that are mediated by oxytocin, the superior temporal sulcus (STS) and the amygdala, have been implicated in ToM processing [27,30]. Specifically, the STS is involved in representing the mental states of other individuals, whereas the amygdala appears to be one of the regions involved in representing the mental states of others as well as the self [30]. It is possible that the enlarged amygdalar volume may result in issues representing the mental states of others and one’s self. However, a single study has associated ASD with reduced amygdalar volumes [31]. Thus, more research is required to examine amygdalar sizes in individuals with ASD compared to healthy subjects.

7.5 Future Directions

The effect of OXTR SNPs on ASD risk and resulting social communication deficits is still unknown. Studies with larger sample sizes, in different ethnicities, that look at specific OXTR SNPs evaluating this relationship are needed. In addition, the formation of haplotypes between specific OXTR SNPs should be accounted for in future studies. However, the rs2254298 marker has repeatedly been shown to be associated with ASD risk [1,2] and social communication deficits.

Research within the rs2254298 marker, and other SNPs that consistently emerge from on-going research, should move towards a focus of understanding their functionality within the brain. As described above, this marker has been implicated in enlarged amygdalar volumes, which is an area in the brain mediated by oxytocin that has subsequently been associated with the ToM hypothesis of ASD. Thus, findings from studies, such as the current investigation, are important as they may provide support for hypothesis surrounding the aetiology of ASD derived from different disciplines. Future research is also needed to examine the amygdalar sizes in individuals with ASD, individuals with ASD secondary to a
known cause, and healthy subjects. It is important to note that the ToM hypothesis of ASD has limitations. Specifically, the social behaviours that are characteristic of ASD are present prior to the emergence of the earliest precursors to the theory [32]. A second theory, known as the social motivation theory of ASD, has recently gained support [33]. This theory also implicates the amygdala, and other neural regions mediated by oxytocin (such as the ventral striatum), as being affected in the brains of individuals with ASD [33].

Another potential avenue for future research includes the evaluation of potential gene-gene and gene-environment interactions. Previous research has indicated that the rs7632287 marker is involved in transcription factor binding [11,34]. It has yet to be experimentally shown that this genetic variant, or other SNPs within the OXTR, are involved in altering gene expression in vivo indicating a potential area for future research [11,34]. Alterations in gene expression, also termed epigenetics, can occur during cell proliferation or throughout development [35]. Epigenetic change can result from either genetic or environmental variations or a combination of both [36,37]. Most importantly, environmental factors occurring prenatally or throughout lifetime are known to influence epigenetic markers resulting in the silencing different genes.

### 7.6 Conclusions

The purpose of this thesis was to evaluate the relationship between OXTR SNPs, and ASD and social communication deficits. Three conclusions were drawn from this thesis. First, no associations were found between any genotype provided by the four SNPs and ASD after adjusting for multiple comparisons. This is surprising given that previous studies have demonstrated a significant association for each of these SNPs in smaller samples sizes [6-10]. Second, the rs2254298 marker was found to be significantly associated with higher social communication scores in verbal participants (see Table 5.4 in Chapter 5). This indicates that these individuals have more severe social communication deficits. Amygdala
enlargement has been previously related to severity of social and communication impairments in toddlers with ASD [38]. An enlarged amygdala may account for the reduction in oxytocin resulting from the presence of SNPs, such as those at the rs2254298 marker, within the OXTR. Finally, the rs7632287 marker showed a potential association ($p_{\text{mixed}} = 0.1096; p_{\text{Caucasian}} = 0.0884$) with lower social communication scores. This implies that this SNP is associated with fewer social communication deficits. This is in contrast to previous research in the area. Though it is possible that epigenetic mechanisms are involved in the mediating this SNP representing a major area for future research.

Taken together, these findings provide support for the idea that specific genetic variants are responsible for mediating different symptom domains rather than overall risk for ASD. Further examination should focus on evaluating the relationship between OXTR SNPs and social communication deficits in different ethnicities while also examining potential haplotype formations. In addition, epigenetic and gene-environment interactions should be examined to provide further insight into the overall aetiology of ASD. In conclusion, the findings from these studies provide limited support for the role of OXTR SNPs in ASD. The clinical significance of these associations remains unknown, however, it is likely that these associations do not play a role in the severity of symptoms associated with ASD. Rather, they may be important in the appearance of social deficits due to the rs2254298 markers association with enlarged amygdalas that in turn results in a more severe symptomatology.
7.7 References


Appendix A

Participant Breakdown

A.1 Objective One

The first objective was to compare the genotype distribution of four OXTR variants in individuals with ASD to those in their unaffected siblings. *Figure A1* outlines how the final sample for this objective was reached.

*Figure A1* Eligibility criteria for objective one. Cases were eligible for this study if they had a confirmed diagnosis of ASD without a known genetic disorder (i.e. Fragile X) or neurodevelopmental disorder (NDD; i.e. ADHD), were from a simplex family, had an unaffected sibling older than three in the Registry, and were older than three at the time of last contact.

120
A.2 Objective Two

The second objective was to determine the association between specific OXTR genotypes and social communication deficits among individuals with a diagnosed ASD. Figure A2 outlines the sample after the eligibility criteria has been employed.

Figure A2 Eligibility criteria for objective two. Cases were eligible for this study if they had a confirmed diagnosis of ASD without a known genetic disorder (i.e. Fragile X) or neurodevelopmental disorder (NDD, i.e. ADHD), had an ADI-R score, were from a simplex family, and were older than three at the time of last contact.
Appendix B

Experimental Conditions

**Table B1** Experimental conditions for genotyping rs2254298, rs53576 and rs1042778

<table>
<thead>
<tr>
<th>Component</th>
<th>Per Reaction</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.73</td>
<td>*Using TaqMan Genotyping Master Mix</td>
</tr>
<tr>
<td>TaqMan MasterMix (2x)</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>40X SNP Assay Mix</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1.50</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Cycling Conditions (ABI thermocycler)**

| Hold                   | 50C          | 2 minutes                                  |
| Hold                   | 95C          | 10 minutes                                 |
| Denature               | 95C          | 15 seconds 40 cycles                      |
| Anneal/Extend          | 60C          | 1 minute 40 cycles                         |

**Scanning Conditions (ViiA7)**

| Hold                   | 60C          | 30 seconds                                 |

**Table B2** Experimental conditions for genotyping rs7632287

<table>
<thead>
<tr>
<th>Component</th>
<th>Per Reaction</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.95</td>
<td>*Using TaqMan Universal Master Mix II with UNG</td>
</tr>
<tr>
<td>TaqMan MasterMix (2x)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>40X SNP Assay Mix</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2.00</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Cycling Conditions (ABI thermocycler)**

| Hold                   | 50C          | 2 minutes                                  |
| Hold                   | 95C          | 10 minutes                                 |
| Denature               | 95C          | 15 seconds 40 cycles                      |
| Anneal/Extend          | 60C          | 1 minute 40 cycles                         |

**Scanning Conditions (ViiA7)**

| Hold                   | 60C          | 30 seconds                                 |
Appendix C

ADI-R Scoring Scheme for Social Communication Deficits

*Table C1* Scoring scheme for the ADI-R interview based on the clinician’s judgment

<table>
<thead>
<tr>
<th>Score</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Behaviour is not present</td>
</tr>
<tr>
<td>1</td>
<td>Infrequent abnormal behaviour but not severe enough to meet the criteria for 2</td>
</tr>
<tr>
<td>2</td>
<td>Abnormal behaviour</td>
</tr>
<tr>
<td>3</td>
<td>Extremely abnormal behaviour</td>
</tr>
</tbody>
</table>
Appendix D

Linkage Disequilibrium Calculation

D.1 Linkage Disequilibrium

The term linkage disequilibrium (LD) refers to the non-random association of alleles at different loci [1]. LD is a common approach in population genetics that is used for a variety of different applications [1,2]. For example, it can be used to finely-map disease genes after localization, as well as to examine the relationships between different SNPs to detect alleles involved in disease risk [2]. The $r^2$ score ranges from 0 to 1 with a score of 1 indicates that the two markers provide the exact same information [1]. Scores greater than 0.5 are indicative of a strong LD, scores between 0.2 and 0.5 represent a moderate LD, and scores lower than 0.2 indicate a weak or no LD [1]. Table D1 provides the results from a LD analysis of the four OXTR variants.

Table D1 Linkage disequilibrium calculations for the four OXTR variants

<table>
<thead>
<tr>
<th>Variant 1 (RS #)</th>
<th>Variant 2 (RS #)</th>
<th>$r^2$</th>
<th>D</th>
<th>Dprime</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs7632287</td>
<td>rs1042778</td>
<td>0.0557</td>
<td>0.0492</td>
<td>0.3153</td>
</tr>
<tr>
<td>rs7632287</td>
<td>rs2254298</td>
<td>0.0356</td>
<td>-0.0252</td>
<td>0.9502</td>
</tr>
<tr>
<td>rs1042778</td>
<td>rs2254298</td>
<td>0.0415</td>
<td>-0.0304</td>
<td>0.7669</td>
</tr>
<tr>
<td>rs7632287</td>
<td>rs53576</td>
<td>0.0002</td>
<td>0.0026</td>
<td>0.0288</td>
</tr>
<tr>
<td>rs2254298</td>
<td>rs53576</td>
<td>0.0139</td>
<td>0.0175</td>
<td>0.4535</td>
</tr>
<tr>
<td>rs1042778</td>
<td>rs53576</td>
<td>0.0827</td>
<td>0.0667</td>
<td>0.4979</td>
</tr>
</tbody>
</table>

All potential pairings of SNPs exhibited weak or no LD indicating that the markers are in equilibrium (randomly associated). Previous research in the area implicated six different potential LD blocks (greater than two SNPs in a pair) within the OXTR that have contained the rs2254298 and rs53576 markers [3]. The results from the current investigation suggest that not evaluating the different potential haplotype formations between these four variants may not have been a limitation with this thesis. This is because SNPs that are in LD are more likely to form haplotypes.
D.2 References


Appendix E

Ethics Approval

QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS
RESEARCH ETHICS BOARD (HSREB)

HSREB Initial Ethics Clearance

August 27, 2015

Ms. Meagan Milton
Department of Public Health Sciences Queen’s University

ROMEO/TRAQ: #6016257
Department Code: EPID-527-15
Study Title: The differential impact of oxytocin receptor (OXTR) genotypes on the risk of autism spectrum disorders and resulting social communication deficits.
Co-Investigators: Mrs. H. Ouellette-Kuntz, Dr. X. Liu
Review Type: Delegated
Date Ethics Clearance Issued: August 27, 2015
Ethics Clearance Expiry Date: August 27, 2016

Dear Ms. Milton,

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the application and granted ethics clearance for the documents listed below. Ethics clearance is granted until the expiration date noted above.

- Protocol:

Documents Acknowledged:

- CORE Certificate – M. Milton
**Amendments:** No deviations from, or changes to the protocol should be initiated without prior written clearance of an appropriate amendment from the HSREB, except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

**Renewals:** Prior to the expiration of your ethics clearance you will be reminded to submit your renewal report through ROMEO. Any lapses in ethical clearance will be documented on the renewal form.

**Completion/Termination:** The HSREB must be notified of the completion or termination of this study through the completion of a renewal report in ROMEO.

**Reporting of Serious Adverse Events:** Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events must be reported within 15 days after becoming aware of the information.

**Reporting of Complaints:** Any complaints made by participants or persons acting on behalf of participants must be reported to the Research Ethics Board within 7 days of becoming aware of the complaint. **Note:** All documents supplied to participants must have the contact information for the Research Ethics Board.

Investigators please note that if your trial is registered by the sponsor, you must take responsibility to ensure that the registration information is accurate and complete.

Yours sincerely,

\[Signature\]

Chair, Health Sciences Research Ethics Board

*The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations, Canadian General Standards Board, and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Federalwide Assurance Number: FWA#:00004184, IRB#:00001173

HSREB members involved in the research project do not participate in the review, discussion or decision.*