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Effect of the Ketogenic Diet on Behavioral Symptoms of Autism in the Poly(IC) Mouse Model

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EFFECT OF THE KETOGENIC DIET ON BEHAVIORAL SYMPTOMS OF AUTISM IN THE POLY(IC) MOUSE MODEL

BY

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ABSTRACT

Autism spectrum disorder (ASD) is a neurological disorder characterized by decreased sociability, deficits in communication, and restricted and repetitive behaviors. The ketogenic diet (KD), a high-fat, low-carbohydrate, and moderate-protein diet has been shown to improve these three behavioral symptoms in the BTBR mouse model of autism. However, further research is required to strengthen the body of knowledge surrounding the potential of KD as diet therapy for autism. Epidemiological observations have shown that maternal immune activation (MIA) during pregnancy increases the risk of autism in offspring. Based on these observations, the polyinosinic:polycytidylic acid (poly(IC)) mouse model was developed as an animal model to study autism. Poly(IC) is a synthetic analog of double stranded RNA and acts as a viral mimic. It is injected into a pregnant dam, activating an immune response without causing an infection. The offspring of this protocol are the poly(IC) MIA mouse model. They have been shown to have the autistic symptoms of deficits in sociability and communication as well as increased repetitive behaviors. In this study, pregnant dams were injected with poly(IC) or the saline vehicle during the late first trimester. The offspring were separated into control and test groups. At 5 weeks of age, the test group was placed on a 6:1 fat:(carbohydrates + protein) KD while the control groups remained on standard chow. After three weeks on the diet, we assessed sociability, repetitive behavior, and communication. Our results showed that KD reversed increased self-grooming in poly(IC) mice. Results did not indicate autistic-like behavior in our poly(IC) mice for social contact, sociability, grooming during the 3-chamber test, or repetitive behavior in the marble-burying test. However, KD increased social contact in poly(IC) mice. It also increased sociability and decreased 3-chamber grooming in poly(IC) males. Poly(IC) mice did not have a deficit in the social transmission of food preference task, a previously unused assessment of the poly(IC) mouse model. While our study did not succeed in replicating several autistic behaviors in the poly(IC) mouse model, KD had influence on behavior in multiple measures, increasing sociability and decreasing grooming. This suggests that KD may be an effective diet therapy for autism.

INTRODUCTION

Autism Spectrum Disorder Definition & Diagnosis

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders characterized by deficits in social communication and interaction as well as repetitive and restricted behaviors. While instances of ASD are often referred to simply as autism, these disorders incorporate a wide range of symptoms which additionally can range from mild to severe. ASD includes multiple disorders such as autism, Asperger syndrome, childhood disintegrative disorder, and pervasive developmental disorders-not otherwise specified (PDD-NOS) (Kim & Lord, 2013).

Diagnostic criteria in the Diagnostic and Statistical Manual of Mental Disorders (DSM V) of the American Psychiatric Association (APA) currently include the presentation of social communication and interaction impairments and repetitive and restricted behaviors in early development. Diagnosis generally occurs on average around 4 years of age and is still entirely behaviorally based as there are currently no reliable biological markers for autism (Kim & Lord, 2013). Later in life, these symptoms cause significant impairment in the social or occupational life of the patient (APA, 2013). Other symptoms can include intellectual, language, sensory, and motor impairments (Kim & Lord, 2013).

Impact & Prevalence of ASD

ASD can severely compromise the quality of life in children and adults with the disorder. Their behaviors—which can include complex rituals, tantrums, and even self-injury—can be highly disruptive to daily life. The detriment to relationships, school, and employment can have lasting effects on the life trajectory of autistic individuals. Patients often do not achieve full independence (Lee *et al.*, 2008). Medical expenditures for autistic

children are significantly higher than they are for their healthy peers. The lifetime cost of autism was estimated in 2007 to be $3.2 million (Ganz, 2007). In the UK, the public cost of supporting children was estimated in 2009 to be £2.7 billion annually and, for adults, £25 billion (Knapp *et al.*, 2009).

The Centers for Disease Control and Prevention reported that the overall prevalence of ASD in 2010 was estimated to be about 1 in 68 among 8 year olds in the United States. This has increased from a rate of 1 in 110 in 2006. In the past 40 to 50 years it has been estimated that rates of autism have increased by at least a factor of 20. The ratio of ASD diagnosis differs significantly between the sexes at a ratio of approximately 4.5 males to 1 female. The factors leading to the increase in rates of autism are unclear and likely multifactorial, including greater capabilities of detection, heightened awareness, and an increase in risk factors or etiology (CDC, 2014; Currenti, 2010). Interestingly, 3-25% of individuals eventually lose their ASD diagnosis. Different types of ASD or inaccurate diagnosis could explain these findings. Additionally, effective treatment may maximize the likelihood of children achieving this optimal outcome (Helt *et al.*, 2008; Fein *et al.*, 2013).

TREATMENT OF ASD

Unfortunately, there is no cure for ASD and the treatments for the disorders are limited. The most effective treatment options for ASD include the combination of educational programming and behavioral intervention. In this treatment, patients undergo training for speech, language, social skills, and behavior. Ideally such therapy and education begins as early as possible. Such treatment requires frequent intensive sessions with trained professionals, making it very expensive and time-consuming. Additional treatment options include addressing the non-core symptoms of ASD such as motor therapy for motor

deficits and risperidone or aripiprazole for irritability, as well as medications for inattention, hyperactivity, sleep problems, anxiety, mood, and aggression (Lofthouse *et al.,* 2012). Treating comorbidities such as seizures, gastrointestinal, metabolic, and hormonal disorders can also help a patient (Bauman, 2014). However, medications for these symptoms are often less effective for children with ASD than they are for those without and they often come with serious side effects. Other complementary and alternative treatments include diet intervention, meditation, therapy animals, chelation therapy and music therapy. But many of these treatments are still unsubstantiated (Lofthouse *et al.,* 2012; Matson *et al*., 2013).

NEUROANATOMY & ETIOLOGY OF ASD

There are currently no consistent neuroanatomical pathologies confirmed for ASD. However, the characteristic symptoms of ASD suggest involvement of the frontal lobe, superior temporal cortex, the parietal cortex, amygdala, orbitofrontal cortex, caudate nucleus, and language cortices (Amaral *et al.,* 2008). Various structures have been implicated in postmortem and structural magnetic resonance imaging. Differences have been seen in the volume of the total brain, cerebral hemispheres, cerebellum, caudate nucleus, corpus callosum, and amygdala (Amaral *et al.*, 2008; Stanfield *et al.*, 2008). Overall, the brain of a child with ASD is enlarged relative to controls during early postnatal life and then growth decreases relative to controls in adolescence. Studies have suggested that the abnormal enlargement of the amygdala is correlated with heightened anxiety. However, these studies have been limited due to factors such as small sample size, comorbidities, and the inability to confirm diagnosis until 2-3 years of age (Amaral *et al.*, 2008; Stanfield *et al.*, 2008).

The cause of autism is still largely unknown and no reliable biomarkers have been identified. ASD is likely a disorder resulting from a combination of genetic and environmental factors. ASD can be caused by known genetic syndromes, and this is the case for approximately 15% of instances of ASD and typically known as syndromic autism (Gabrucker, 2013). Fragile X syndrome, tuberous sclerosis, neurofibromatosis, untreated phenylketonuria, Angelman, Cornelia de Lange, and Down syndrome can all present autistic features. The most common of these are Fragile X syndrome and tuberous sclerosis which both increase protein synthesis (Laumonnier *et al*., 2004; Persico & Napolioni, 2013). Autism is strongly associated with mutations in genes which encode for proteins that play a role in regulating synaptic protein synthesis (Persico & Napolioni, 2013).

Indeed, a cross-sectional study of 210 dizygotic twins and 67 monozygotic twins under the age of 18 revealed that rates of ASD concordance between dizygotic and monozygotic twins are 31% and 88%, respectively. The 4:1 male to female ratio of incidence suggests there is a sex-linked genetic influence. The possession of two X chromosomes may provide protection for females. Interestingly, all 9 pairs of female monozygotic twins in the study had a concordance of 100% while the 113 dizygotic twin pairs containing at least one female had a concordance of only 20% (Rosenberg *et al.*, 2009). Maternal environment during pregnancy is also considered a factor due to a higher concordance rate between dizygotic twins than between siblings (3-14%) (Sumi *et al.*, 2006). A study by Schwartzer *et al*. found that in mouse models of autism, prenatal exposure to maternal immune activation in genetic mouse models of autism exacerbated their autistic symptoms, further suggesting an interaction between genetic and environmental factors in the etiology of autism (2013).

Maternal Environment

Maternal environment is associated with a number of developmental neurological disabilities and disorders. Approximately 1 in 33 U.S. children are born with a birth defect. Only about 25% of these congenital abnormalities can be attributed to solely genetic causes. It is estimated that about 15% of developmental disorders are the result of environmental factors alone. The remaining 60% of disorders are multifactorial combinations of genetic and environmental causes or have currently unknown origins (Czeizel, 2005).

In the case of ASD, there are a number of associated maternal environment risk factors. Abnormalities in fetal growth, whether above or below standard are associated with greater ASD risk (Abel, 2013). Furthermore, risk of ASD was decreased by approximately 40% in mothers who took prenatal supplements at least 4 times per week during the preconceptional window (3 – 1 months before conception) (Schmidt *et al.*, 2011). Taking valproic acid, an antiepileptic, during the prenatal period has been shown to increase risk of ASD behaviors in humans and animal models. (Engel & Daniels, 2011; Ornoy, 2009; Dean *et al.*, 2002). Zinc deficiency, maternal diabetes, toxins, parental age, prenatal and perinatal stress, and prenatal infection are all events and situations currently recognized as environmental risk factors for ASD (Grabrucker, 2013).

Maternal Immune Activation and ASD

Maternal infection has been associated with risks of various neurodevelopmental disorders, such as schizophrenia, autism, and cerebral palsy (Boksa, 2010). Evidence for the significance of prenatal infection on the risk of ASD is strong and growing. Prenatal viral infection exposure in the first trimester and bacterial infection in the second trimester

correlate with increased risk of ASD diagnosis in offspring (Atladottir *et al.*, 2010). This effect has also been seen in animal models including mice, rats, and non-human primates (Boska, 2010; Bauman, 2014). Mothers with autistic children are more likely to have autoantibodies with negative effects on fetal brain development. It is suggested that these are transferred through the placenta to the offspring (Currenti, 2010). Mothers of autistic children are also more likely to have autoimmune diseases such as rheumatoid arthritis, celiac disease, and type 1 diabetes (Patterson, 2011).

The mechanisms by which maternal immune activation (MIA) increases the risk of ASD is not fully understood, but it is likely multifactorial. Data suggests that this environmental factor combines with genetic risk factors. Indeed, there is an association between being born at the peak of the influenza season and having tuberous sclerosis with autistic behaviors (TSC-ASD) while there is no such association between date of birth and TSC alone (Patterson, 2011). The multiple neurobiological effects MIA has on animal models may help to explain its mechanisms of action. MIA has been shown to increase circulating interleukin-6 (IL6) and placental cytokines. It is known to lead to oxidative stress, morphological changes in the brain, and changes in neurotransmitter systems (Boksa, 2010; Patterson, 2011).

IMMUNE DYSFUNCTION & INFLAMMATION

The influence of MIA is likely connected to the data showing immune dysfunction and inflammation in individuals with ASD. The association between ASD and immune dysfunction and inflammation is the most strongly supported correlations between ASD and a physiological abnormality (Rossignol & Frye, 2012). This relationship is shown in many ways. The immune system of autistic individuals is often abnormal. Families with

autism are more likely to have autoimmune disorders (Croen *et al.,* 2005). Autistic children show serum antibody reactivity against human cortical and cerebellar brain regions (Currenti, 2010). An inflammatory-like state was found in postmortem autism brains: there were elevated cytokines and activated microglia and astrocytes throughout subjects ranging in age from 5 to 44 years, indicating that these changes occur early and are likely permanent (Vargas *et al.,* 2005). Similarly, raised cytokines were also found in the cerebrospinal fluid of living autistic children (Chez *et al.*, 2007). Additionally, autistic subjects have elevated plasma cytokines and chemokines; the blood brain barrier is permeable to these pro-inflammatory mediators (Ashwood, *et al.,* 2011; Li *et al.*, 2009). Inflammation in the gastrointestinal tract has also been indicated, perhaps contributing to the comorbidity between autism and gastrointestinal problems. Furthermore, microarray studies of autistic brains have shown that there is dysregulation of genes relating to immune function (Patterson, 2011).

The interaction between immune dysfunction/inflammation and ASD is not fully understood. Genetic susceptibility to immune-related disorders could alter brain function. However, altered brain morphology could also interfere with immune function, making this dysregulation a symptom rather than a cause. Some genes considered in the etiology of autism regulate both brain and immune development. Such genes include macrophage migration inhibitory factor (MIF), MET encoding tyrosine kinase, the reelin gene (RELN) and the human leukocyte antigen (HLA) genes (Careaga et al., 2012).

POLY(IC) MOUSE MODEL

In order to further investigate MIA as a risk for autism, research has developed MIA animal models of autism. Many animal modeling studies have shown that acute and lasting changes to behavior and CNS structure and function can be caused by prenatal immune activation. These studies, however, have varied regarding the materials, species, methods, and measurements used (Boksa, 2010). Both rodent and non-human primate models have exhibited abnormal, ASD-like behavior as a result of MIA (Boska, 2010; Bauman, 2014). Agents used for creating prenatal immune activation can cause an antiviral response, such as in the case of polyinosinic:polycytidylic acid (poly(IC)), or they can cause an antibacterial response, such as in the case of lipopolysaccharide (LPS) (Patterson, 2011).

This thesis investigated the effectiveness of dietary treatment for ASD using the poly(IC) mouse model. Offspring of dams injected with poly(IC) have been found to exhibit the core deficits of autism: decreased social and communicative behavior as well as increased repetitive and stereotyped behavior (Malkova *et al.*, 2012). The mechanism by which poly(IC) has this effect has been explored. Poly(IC) mimics the structure of dsRNA that results from viral replication. It binds to the Toll-like receptors, dsRNA-activated protein kinase, and other proteins, activating an immune response and a cellular danger response. Poly(IC) inhibits translation of cap-dependent mRNAs and stimulates IL1β, IL6, TNFα, type I interferons (IFN) alpha and beta. Exposure to poly(IC) produces an observable response of initial fever followed by hypothermia as well as weight loss and reduced activity, food intake, and water intake. Recovery occurs approximately 24 hours after exposure (Naviaux *et al.*, 2013; Traynor *et al.,* 2004).

In addition to increasing cytokines and chemokines, poly(IC) also leads to deficits in the mitochondrial function of leucocytes. Adult offspring had significant decreases in mitochondrial ATP production (Arrode-Bruses & Bruses, 2012; Giulivi, 2013). This significant difference in metabolic function suggests that diet therapy may be an effective treatment for the poly(IC) model of ASD.

KETOGENIC DIET AND ASD

The ketogenic diet (KD) is a restricted carbohydrate, sufficient protein, and high fat metabolic therapy. KD has been used effectively as a diet therapy for epilepsy since the 1920s, with an average of 43% of children and adults reaching greater than or equal to a 50% seizure reduction (Payne, 2011). It has been shown to have various beneficial effects for many neurological diseases, including headache, neurotrauma, Alzheimer's disease, Parkinson's disease, sleep disorders, brain cancer, pain, multiple sclerosis, and depression (Stafstrom & Rho, 2012). ASD and epilepsy are highly comorbid, and it is suggested that KD may be an effective therapy for ASD symptoms (Kim & Lord, 2013; Ruskin *et al.*, 2013).

Indeed, therapeutic effects of KD have been shown for autism (Ruskin *et al.*, 2013). A BTBR mouse model, which exhibits impaired sociability and communication as well as increased repetitive and restricted behaviors, was fed a strict KD (6.6:1 fats:(carbohydrates + proteins)). The diet alleviated all three of the core behavioral deficits (Ruskin *et al*., 2013).The mechanisms by which KD has neuroprotective and therapeutic effects are not yet fully understood, but there are several properties of the diet that may provide explanation for its potential use with ASD.

KD has anti-inflammatory properties which may relieve inflammation and inflammation-related symptoms in ASD (Yang & Chenge, 2010; Kim *et al.,* 2007; Gasior,

2006; Cullingford, 2004). It also reduces thermal nociception in juvenile and adult rats (Ruskin *et al*., 2009). A suggested mechanism for this anti-inflammatory effect is that saturated fatty acids, the predominant energy fuel for a body on the ketogenic diet, contribute to the regulation of inflammatory response genes by activating toll-like receptors that activate nuclear factor-kappa B and cycloocygenase-2 (Forsythe *et al.*, 2008). In a study examining a murine model of experimental autoimmune encephalomyelitis, KD decreased inflammatory cytokines and chemokines while also improving motor disability, spatial learning and memory, and long-term potentiation in the CA1 hippocampus region (Hao *et al.,* 2012).

The influence of the ketogenic diet on metabolism is also likely key for the beneficial effects of the diet. Metabolic disorders are common in autism. Mitochondrial electron transport chain complexes have decreased expression in the cerebellum and frontal and temporal regions in children with autism, likely contributing to abnormal metabolism and oxidative stress (Chauan *et al.*, 2011). KD switches the body's energy source from glucose to ketones, fundamentally altering metabolism.

One suggested metabolic mechanism by which the ketogenic diet may be effective is through adenosine. Adenosine is a neuromodulatory purine that acts as a sleep modulator and anticonvulsant and is a core component of ATP (Masino *et al.*, 2011). Additionally, adenosine kinase inhibitor and adenosine deaminase inhibitor have antinociceptive and anti-inflammatory effects (Poon & Sawynok, 1999). Those with ASD, as previously mentioned, often suffer from comorbidities of sleep disorders and epilepsy and ASD is highly associated with inflammation (Masino *et al.,* 2011). Variants of adenosine A2A receptor gene have been associated with increases in autistic symptoms (Freitag *et al.*,

2009). Additionally, autistic children have been reported to have reduced adenosine deaminase activity (Bottini *et al.,* 2001). KD has been shown to increase ATP and adenosine in the brain (Nakazawa *et al*., 1983; Masino *et al.*, 2009). Ketone bodies are increased while on the ketogenic diet. These ketone bodies substitute for glucose and are more efficient in ATP production (Masino *et al.,* 2009).

The present study will investigate the ability of the ketogenic diet to alleviate the autistic symptoms in the poly(IC) mouse model of ASD, further illuminating the therapeutic relevance of KD for ASD.

THESIS OVERVIEW

The current study aims to investigate the effect of the ketogenic diet on the poly(IC) mouse model of autism in order to further the body of knowledge regarding the applications and efficacy of ketogenic diet therapy. Beneficial effects of the ketogenic diet were found previously in the BTBR strain of mice which, however, have an undefined genetic etiology of autism; therefore we chose to use this environmental model. It is hypothesized that the ketogenic diet will relieve autistic behaviors within the poly(IC) mouse model.

MATERIALS AND METHODS

ETHICS STATEMENT

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Trinity College (A3869-01).

ANIMALS

Adult female C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were determined to be "proven breeders" after having one or more previously successful litters. These breeders were housed socially with same-sex cage mates. Estrous cycle was visually monitored daily for each mouse. When a breeder was determined to be in proestrus/estrus phase, she was housed overnight with an adult male C57Bl/6 mouse. The following morning, the female mouse was checked for the presence of a vaginal plug, which marked embryonic day 0.5 (E0.5). Pregnant females were housed socially and not disturbed except for weekly cage cleaning.

On day E10.5, dams were weighed and assigned to either the poly(IC) or saline injection group. Injections of 5mg/kg poly(IC) (potassium salt; P9582; Sigma, St. Louis, MO) or saline were administered intraperitoneally on days E10.5, E12.5, and E14.5. Poly(IC) was supplied by the manufacturer at 10% of the total weight of the salt, and the dosage was based on the weight of poly(IC) itself. All pups from each litter remained with the mother postnatal day 21 (P21), at which time they were weaned and housed socially with same-sex littermates. In the case of only one male or female pup in a litter, the pup was house socially with same-sex, same-age untreated C57 pups.

Between P21 and 5 weeks of age, all offspring were fed control diet (CD; LabDiet 5001, W.F. Fisher & Son, Somerville, NJ). At 5 weeks of age, poly(IC) mice were separated into control and test groups and were either kept on CD or switched to KD (F3666; BioServ, Frenchtown, NJ), respectively. Mice of the saline-treatment group were kept on CD. A saline-treatment group on KD was not included because previous testing has shown KD has no significant effect on behavior in C57Bl/6 control mice (Ruskin *et al.*, 2013). All testing occurred at 8-10 weeks of age at 3-5 weeks of dietary treatment.

BEHAVIORAL TESTING

SOCIABILITY

Sociability was testing using the three-chamber sociability test. A 22 x 42.5 x 19 cm Plexiglas box divided into three equal chambers was used. A 6 x 6 cm door in each of the internal walls allowed for free movement between the chambers. A small cylindrical wire cage (diameter 10.4 cm, height 11 cm, bar intervals 1 cm) was placed in both side chambers. Test subject mice were first habituated to the testing room for 30 minutes and then habituated to the central chamber for 10 minutes with the doors closed. Testing occurred in three 10 minute phases in which the test mouse was allowed to roam freely between chambers (Fig. 1). The test mouse was placed in the central chamber and the doors were lifted at the start of the phase. In the first phase, both wire cages were empty, allowing for a test of side bias. In phase two, a "stranger" mouse (C57Bl/6) was placed in the wire cage of one side chamber, allowing for a test of sociability. In the third phase a novel "stranger" mouse was placed in the other wire cage to allow for a test of preference for social novelty. The placement of the initial stranger mouse was counterbalanced between tests. Stranger mice were sex- and diet-matched to the test mouse, having started

the matched diet several days before testing to account for diet-related olfactory cues. In each phase, mice activity was video recorded and scored for time spent in each chamber and social contact (nose/face/forepaw contact with the cages and/or the stranger mice).

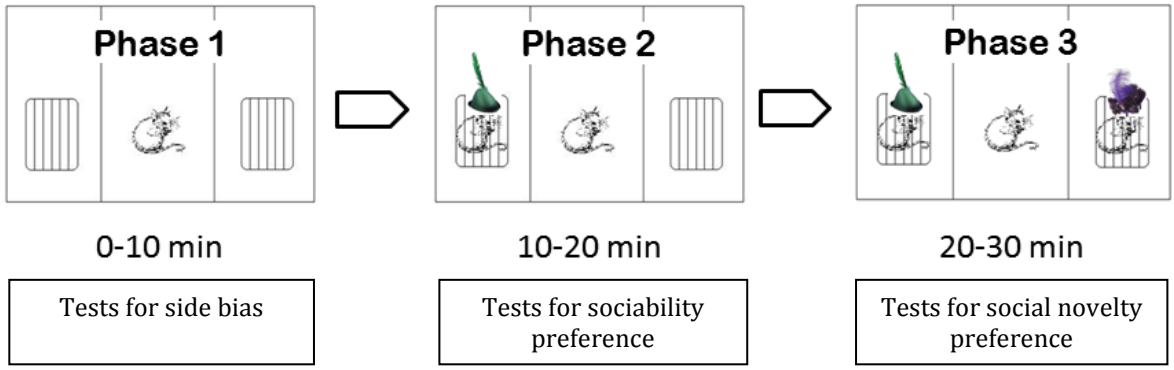

Figure 1. Three-Chamber Test Paradigm.

REPETITIVE & COMPULSIVE BEHAVIOR

Self-grooming was quantified in the three-chamber sociability testing and during a separate 10 minute test in order to measure self-directed repetitive behavior. In this test, the mouse was habituated to the room for 30 minutes and then habituated to the experimental polypropylene cage (19 x 29 x 12.5 cm) for 10 minutes. Mouse behavior was video recorded and quantified in a 10 minute phase following habituation.

Compulsive behavior was measured using the marble-burying test. Mice were allowed to habituate to the room for 30 minutes. An experimental polypropylene cage (19 x 29 x 12.5 cm) was prepared with leveled 5 cm deep clean wood chip bedding (Betachip, Charles River). 15 black glass marbles (1.5 cm diameter) were evenly spaced in a 5 x 3 grid on top of the bedding. After habituation, the test mouse was placed in the experimental cage. After 30 minutes, the mouse was removed and returned to its home cage. A digital picture of the experimental cage was taken from above. Photoshop was used to count

marble pixels, using the "quick selection" tool, and to measure the bedding area, using the "polygonal lasso" tool. Marble pixels were expressed as a percent of the total bedding area pixels. A lower percentage of marble pixels is interpreted as indicating more burying and thus greater compulsive behavior.

Social Communication

Social communication was measured using the social transmission of food preference (STFP) test. Mice were habituated to eating KD or powdered CD, as appropriate, from clear glass jars (Dyets, Inc., Bethlehem, PA) 24 hours prior to the beginning of testing. The round jars had perforated lids, allowing for access to food and diminishing the displacement of food from mice digging. To begin the testing, a demonstrator mouse was removed from the home cage and placed in solitary housing. Both the demonstrator mouse and cage mates then fasted for 18 hours. A feeding jar containing a weighed amount of flavored powdered control diet food ("trained" flavor; 1% cinnamon or 2% cocoa) was presented to the demonstrator mouse and remained in the cage for 2 hours. If the demonstrator mouse had not yet eaten >0.5g of food, the diet was returned to the demonstrator. Once the demonstrator consumed >0.5g of food, it was returned to the home cage and allowed to interact with the cage-mate observer mice for 30 minutes. Observer mice were then separated into individual cages with a jar of the "trained" flavor and a jar of the "untrained" novel flavor. Jars were weighed before and after a 2 hour period in order to determine the preferred flavor.

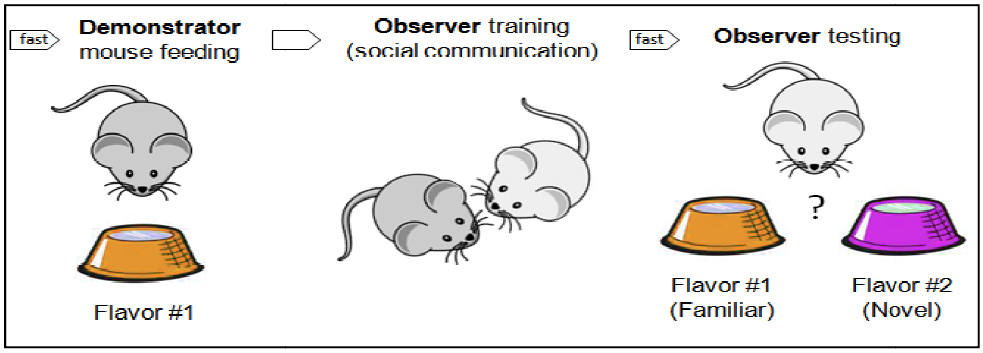

Figure 2. Social Transmission of Food Preference Test Paradigm.

BLOOD ANALYSIS

After the marble-burying test, blood glucose, ketone (beta-hydroxybutyrate, or B-HB) levels were measures. Placed briefly in a plastic container with isoflurane vapors (Animal Health International), mice were anesthetized. Once unconscious, the mouse was removed and the tail vein was punctured with a razor blade. Blood was then analyzed for glucose and ketones using Precision Xtra meters (Abbott Laboratories, Bedford, MA). Mice were returned to their home cage to awake from anesthesia.

STATISTICAL ANALYSIS

Social and grooming behavior videos were scored by two independent researchers, at least one of whom was blind to treatment. Sociability preference in the three-chamber sociability test was defined as the ratio of time spent in the "social" chamber to the total time spent in side chambers. Statistical analysis was conducting using student t-tests or ANOVA with Newman-Keuls post-hoc tests to determine diet, treatment, sex, and interaction effects. When sex differences were not found, data was collapsed across male and female mice. Outliers in each group during a given test were identified using Grubb's outlier test and excluded from analysis. Data are reported as mean +- standard error. $P < 0.05$ was considered significant.

RESULTS

Following 3-5 weeks on their respective diets, the saline-CD, poly(IC)-CD, and poly(IC)-KD groups underwent blood, weight, and behavioral testing in order to investigate the influence of KD on the autistic behaviors of the poly(IC) mouse model.

BLOOD ANALYSIS

Tail blood was collected under anesthesia to measure blood ketones and glucose levels. Poly(IC) mice fed KD had significant blood chemistry changes. For both male and female mice, ketone levels were significantly higher for the KD group compared to the CD groups (Fig. 3A). Within the poly(IC) groups, female mice had significantly higher ketone levels than their male counterparts (Fig. 3A). Mice fed KD also had significantly decreased glucose levels (Fig. 1B). There were no significant glucose differences between sexes.

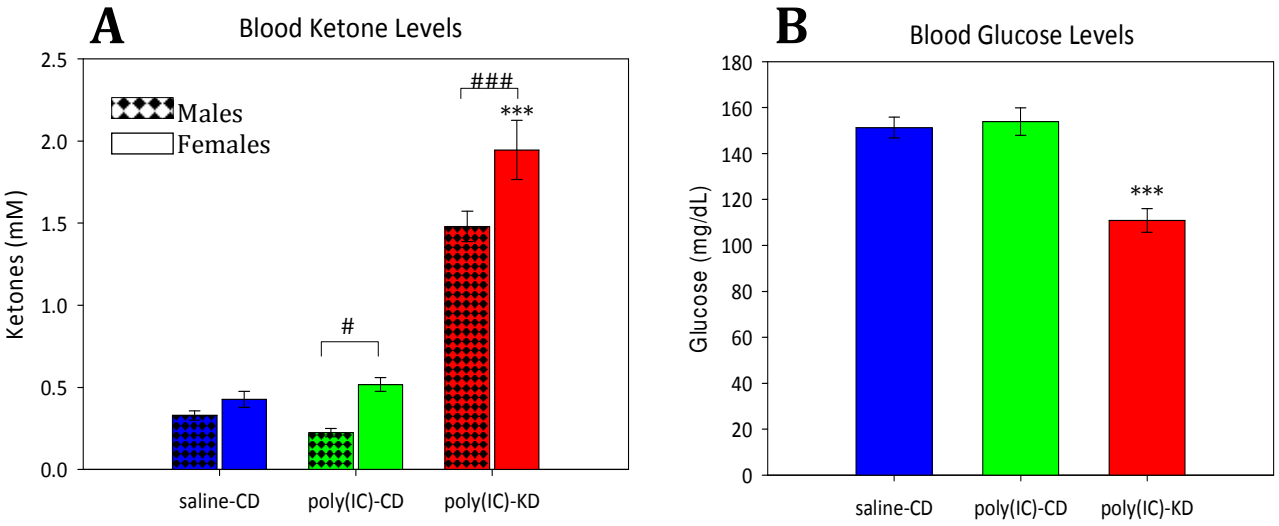

Figure 3. KD increases ketone levels and decreases glucose levels in poly(IC) mice. (**A**) shows ketone levels (mM) for both sexes in each treatment-diet condition. Levels were significantly higher for male and female poly(IC)-KD mice than male and female CD groups. Females in the poly(IC)-KD and poly(IC)-CD groups had significantly higher ketone levels than the males of their treatment-diet group. (saline-CD: male n=7, female n=15; poly(IC)-CD: male n=8, female n=12; poly(IC)-KD: male n=10, female n=11; *** $p<0.001$ between groups, ### $p<0.001$ within group, # $p<0.05$) There was no significant condition x sex interaction. (**B**) shows glucose levels (mg/dL) in each treatment-diet condition. There were no significant sex differences, so data was collapsed. The poly(IC)-KD group had significantly lower glucose that the poly(IC)-CD and saline-CD groups. (saline-CD: n=22; poly(IC)-CD: n=20; poly(IC)-KD: n=21; *** $p<0.001$).

WEIGHT

At 5 and 8 weeks of age, female mice were weighed. As expected, the saline-CD and poly(IC)-CD groups gained significant weight during this 3 week time span. The poly(IC)-KD females, however, did not significantly differ in weight between 3 weeks and 5 weeks of age. Additionally, they weighed significantly less than the CD groups (Fig. 4)

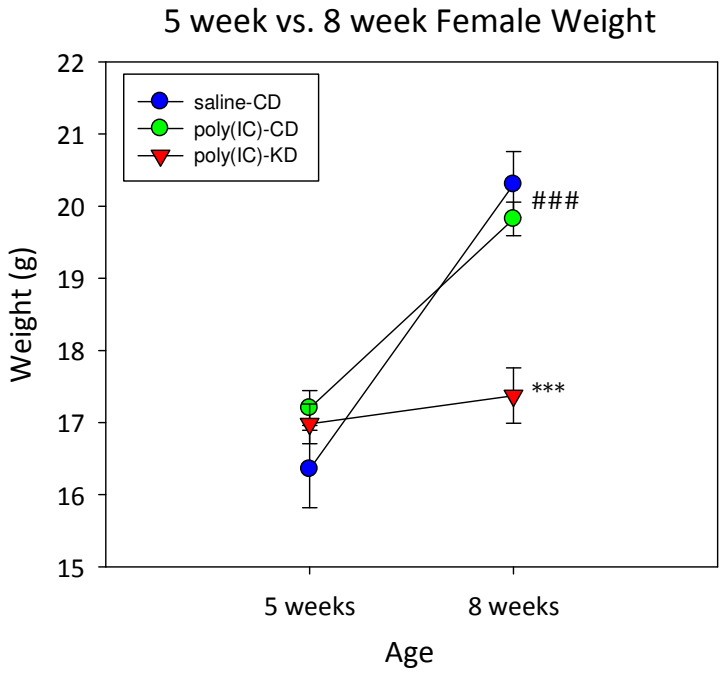

Figure 4. KD decreased weight gain. This figure weights at 3 weeks and 5 weeks of age for female mice in each treatment-diet condition. Saline-CD and poly(IC)-CD mice weighed significantly more at 8 weeks of age than at 5 weeks of age, while poly(IC)-CD mice did not differ in weight significantly between the two ages. Poly(IC)-CD mice weighed significantly less at 8 weeks of age than saline-CD and poly(IC)-CD mice. (saline-CD: n=13; poly(IC)-CD: n=9; poly(IC)-KD: n=11; *** $p<0.001$ between groups, ### $p<0.001$ within group). Data were lost for the poly(IC)-CD male group, so male weight data is not presented.

SOCIABILITY MEASURES

SIDE CHAMBER TIMES

Side chamber times in the 3-chamber test were used to assess sociability. Poly(IC)-KD males spent significantly more time in the target chamber during phase 2 as compared both with their phase 1 scores and with the phase 2 times of both other groups. This demonstrates that poly(IC)-KD males had a preference for sociability while poly(IC)-CD and saline-CD males did not. No differences were found between the groups in the social novelty phase 3 (Fig. 5A). For females, however, no differences were found between the different groups. Each group showed significant preference for sociability in phase 2 and no preference for social novelty in phase 3 (Fig. 5B).

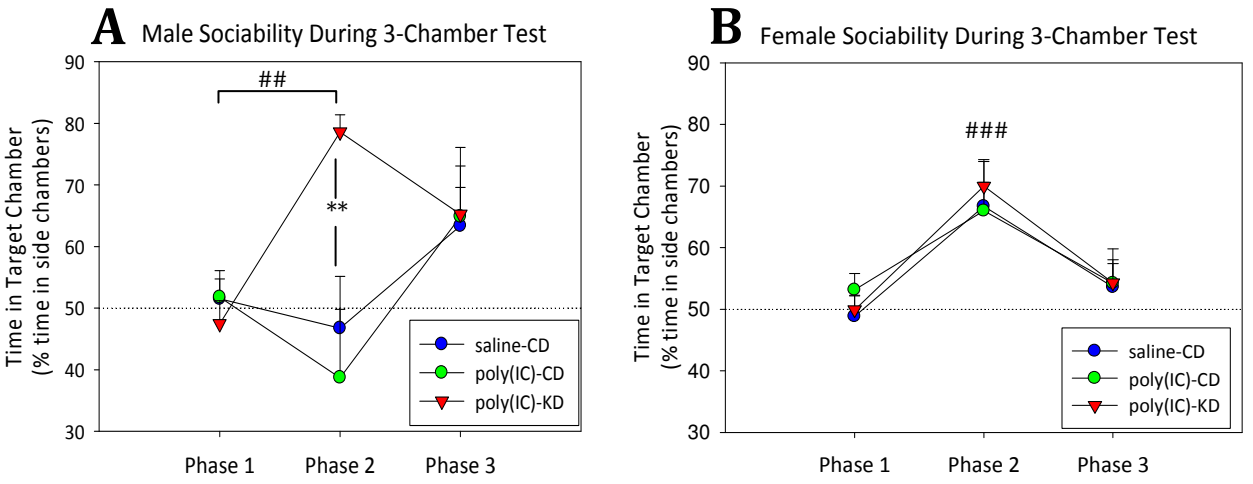

Figure 5. KD increases male poly(IC) sociability during 3-chamber test. (**A**) shows time spent by male mice in the target chamber, which contained a stranger mouse (phase 2) or novel stranger mouse (phase 3), as a percentage of total time spent in side chambers. Male poly(IC)-KD mice spent significantly more time in the target chamber during phase 2 than during phase 1 and were significantly more social in phase 2 than both other groups. (males: saline-CD, n=7; poly(IC)-CD, n=8; poly(IC)-KD, n=8; ** $p<0.01$ between groups, ## $p<0.01$ within group). (**B**) shows time spent by female mice in the target chamber during the three-chamber test. No significant differences existed between groups. Each group spent significantly more time in the target chamber during phase 2 than in either phase 1 or 3. (females: saline-CD, n=12; poly(IC)-CD, n=12; poly(IC)-KD, n=11; ### $p<0.001$ within group).

Social Contact

Social contact made with the stranger mouse during the 3-chamber test was also used to assess sociability. Social contact data yielded different results than data of side chamber times. There were no significant differences between the sexes, so data were collapsed. Time spent by the test mouse making social contact with the target mouse was compared in phase 2 and phase 3. Poly(IC)-KD mice made significantly more social contact than either saline-CD or poly(IC)-CD mice. Similar to 3-chamber data, social contact made by saline- and poly(IC)-CD mice did not differ, demonstrating no deficit in either sociability measure for poly(IC) mice (Fig. 6).

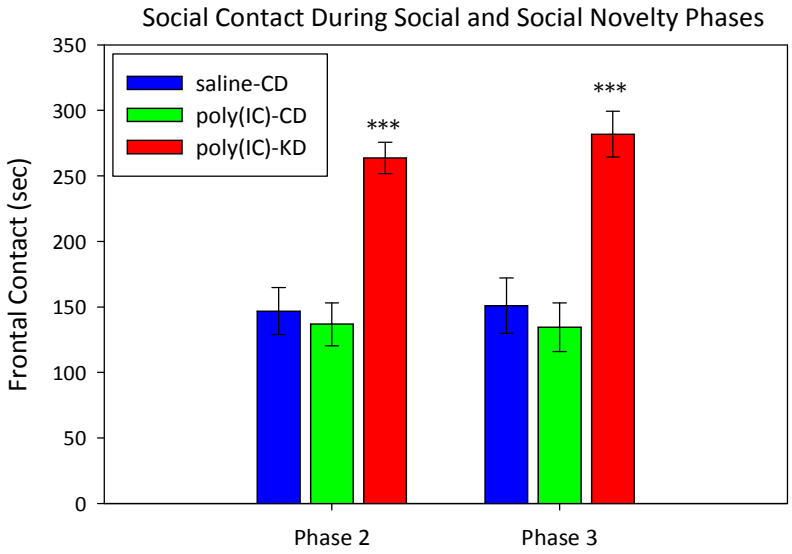

Figure 6. KD increases social contact made by poly(IC) mice in 3-chamber test. Figure shows poly(IC)-KD mice spent significantly more time making social contact with the target mouse's cage in phase 2 and 3 than the other treatment-diet groups. (saline-CD, n=19; poly(IC)-CD, n=18; poly(IC)-KD, n=21; ***p<0.001).

REPETITIVE & COMPULSIVE BEHAVIOR MEASURES

THREE-CHAMBER TEST GROOMING

Time spent self-grooming during phase 1 and 2 of the 3-chamber test was measured to assess self-directed repetitive behavior in a non-social and social setting. A treatment by sex interaction was found. Male saline-CD and poly(IC)-CD mice groomed significantly more in phase 2, the social phase, than in phase 1. Male poly(IC)-KD mice groomed significantly less in the social phase than saline-CD and poly(IC)-CD mice. Additionally, time spent grooming did not differ significantly between the social phase than the non-social phase (Fig. 7A). Each female group groomed significantly more in phase 2 than phase 1, although saline-CD mice showed the most distinct difference. In phase 2 female saline-CD mice groomed significantly more than poly(IC)-CD and poly(IC)-KD (Fig. 7B).

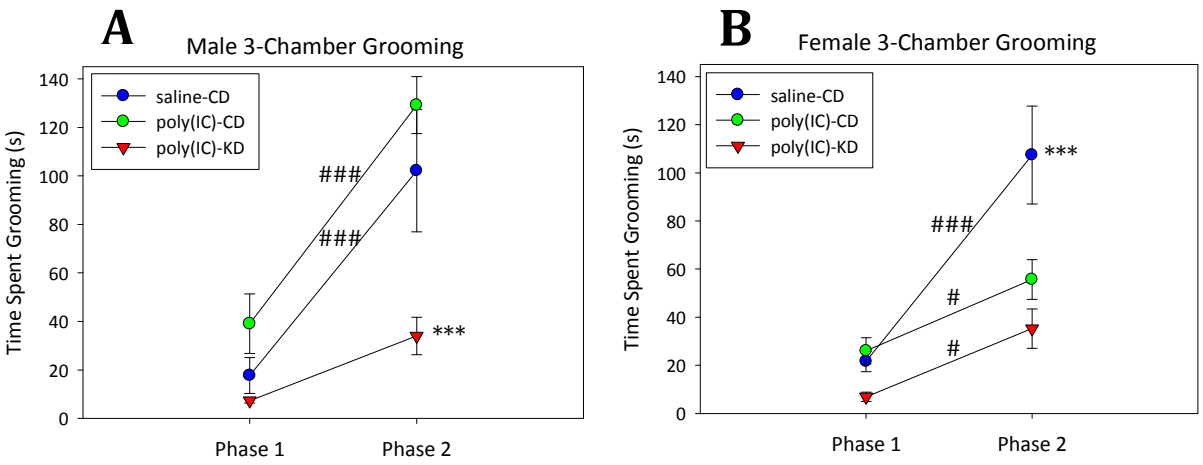

Figure 7. KD lowered grooming in poly(IC) males in the social phase of Three-Chamber Test (**A**) shows time spent self-grooming by male mice during the 1st and 2nd phase of the three-chamber sociability test. Grooming differed significantly between phase 1 and 2 for saline-CD and poly(IC)-CD mice, but not poly(IC)-KD mice. In phase 2 male poly(IC)-KD mice groomed significantly less than the other treatment-diet groups. (males: saline-CD, n=7; poly(IC)-CD, n=8; poly(IC)-KD, n=10; ### $p<0.001$ within group, *** <0.001 between groups). (**B**) shows time spent self-grooming by female mice during the 1st and 2nd phase of the three-chamber test. Grooming differed significantly between phase 1 and 2 for each treatment-diet group. Female saline-CD mice groomed significantly more than the other groups in phase 2. (females: saline-CD, n=11; poly(IC)-CD, n=12; poly(IC)-KD, n=11; ### $p<0.001$ within group, # $p<0.05$).

SINGLE-CHAMBER GROOMING

Single-chamber grooming was also measured to assess self-directed repetitive behaviors. Self-grooming while in a single-chamber was significantly higher for poly(IC)-CD mice compared to the saline and KD groups. Grooming time of the poly(IC)-KD mice did not significantly differ from grooming time of the saline-CD mice. Grooming was significantly increased in offspring by poly(IC) MIA. KD returned grooming to normal levels (Fig. 8). This supports a reversal of a poly(IC)-induced increase of self-grooming by KD.

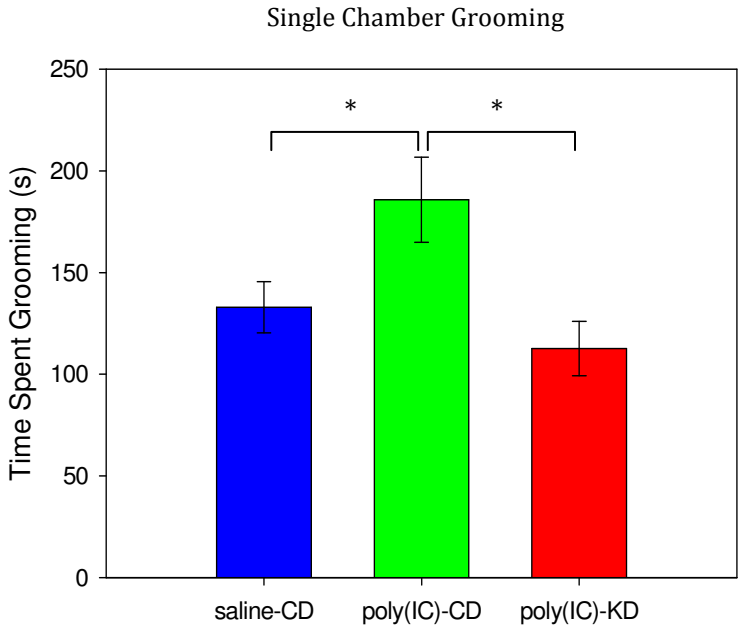

Figure 8. KD reverses increased self-grooming in poly(IC) mice. Figure shows the total time spent self-grooming by the test mouse while in a single chamber. Self-grooming while in a single-chamber was significantly higher for poly(IC)-CD mice compared to the saline and KD groups. Grooming time of the poly(IC)-KD mice did not significantly differ from grooming time of the saline-CD mice. (saline-CD, n=19; poly(IC)-CD, n=20; poly(IC)-KD, n=21; * $p<0.05$).

MARBLE-BURYING

The extent of marble-burying performed by mice during a 30 minute phase was used to assess compulsive behavior. There were no significant differences between any of the groups (Fig. 9).

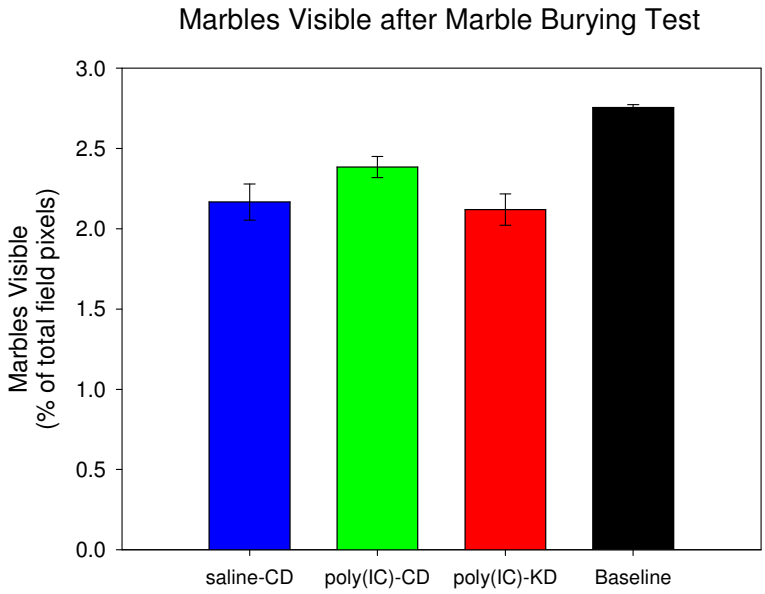

Figure 9. No differences in marble burying between groups. The figure shows the amount of marble pixels visible as a % of the total number of pixels of bedding. The baseline bar shows the amount of marble visible in a test chamber before a mouse is introduced (n=10). No significant differences were found between groups. (saline-CD, n=18; poly(IC)-CD, n=17; poly(IC)-KD, n=21).

SOCIAL COMMUNICATION MEASURES

STFP was conducted to investigate communication. To the best of the author's knowledge, this measure has not been used on the poly(IC) mouse model before. Poly(IC)-CD mice ate significantly more of the trained flavor (0.60±.10g) than the untrained flavor (0.11±.04g) ($n=12$, $p<0.01$). Because no deficit was found in the poly(IC)-CD group, further analysis was not conducted.

DISCUSSION

POLY(IC) MOUSE MODEL OF AUTISM

In this study, blood, weight, and behavioral effects of KD on the poly(IC) mouse model of autism were assessed. As a model for autism, poly(IC) has been previously shown to exhibit core deficits of decreased social and communicative behavior as well as increased repetitive and stereotyped behavior (Malkova *et al.,* 2012). However, analysis of our data showed an unexpected lack of abnormality in our poly(IC) for most behavioral measures. Within the 3-chamber test paradigm, poly(IC) mice did not differ significantly from saline controls for side preference times, social contact times, or grooming times. Furthermore, marble-burying and STFP measures did not indicate a clear deficit for our poly(IC) mice. At this time it is unclear if the lack of a difference between our poly(IC) and saline controls stems from a failure to develop poly(IC) offspring with the full range of autistic symptoms commonly attributed to them or if some abnormality was present in our saline controls, inhibiting proper analysis of our poly(IC) mice.

SOCIABILITY

Several other studies have used the 3-chamber paradigm for sociability and have found different results. A 2012 study by Malkova and colleagues found poly(IC) mice to be significantly less social than their saline control counterparts (Malkova *et al*., 2012). Similarly, Naviaux *et al*. (2013), in the process of investigating antipurinergic therapy for autism features in the poly(IC) mouse, observed that poly(IC) mice were significantly less social in the 3-chamber paradigm both by the side preference and social contact measures. More recently, a study by Xuan & Hampson (2014) found that both males and female subjects had reduced sociability in the 3-chamber test. Each of these studies focused their

attention on the preference for sociability phase 2 portion of the 3-chamber test. Interestingly, what makes our results markedly different from those of these studies is the lack of a significant difference between the control and poly(IC) mice. Similar to other studies, our male poly(IC) mice were considerably anti-social in the second phase of our 3-chamber sociability test, spending only approximately 40% of their time in the chamber with the stranger mouse. However, our saline controls also exhibited anti-social behavior, prohibiting us from characterizing the anti-social behavior of our poly(IC) mice as abnormal.

The difference between male and female mice within our 3-chamber paradigm is not wholly unexpected as general literature is inconsistent regarding the severity of female poly(IC) deficits. While it is unclear why female saline controls would be significantly social in phase 2 while male saline mice were not, the lack of an anti-social characteristic in the poly(IC) females is in agreement with the study by Naviaux and colleagues (2013) which noted milder social abnormalities in their female poly(IC) mice. Other studies however have seen decreased sociability for both males and females (Xuan & Hampson, 2014; Shi *et al.*, 2003; Schwartzer *et al.*, 2013). Overall, there is a lack of research regarding female poly(IC) mice.

Furthermore, the difference in results between the side preference and social contact measures is of interest and requires explanation. Within the 3-chamber test, side preference and social contact are both measures of sociability in the test mouse. Typically the results of each of these measures are similar. Other studies have found that social contact and chamber times are highly correlated; difference between them is mild or non-significant, and the main difference between the two measures is that social contact is a

more sensitive measure of sociability (Silverman *et al.*, 2012; Chadman, 2008; Nadler *et al.*, 2004). Therefore, the apparent difference between our chamber time and social contact data with regard to our female mice (which exhibited very different group effects in the 3-chamber test but were collapsed across sex in the social contact measure) was unexpected. However, in our social contact data, there was a non-significant trend of sex effect ($p=0.1$) with the female saline-CD and poly(IC)-CD groups grooming more than their male peers. The difference of female data suggests that saline-CD and poly(IC)-CD mice spent significant time in the side chamber without making significant contact with the stranger mouse.

Repetitive Behaviors

The measured levels of repetitive, stereotypical behaviors in our poly(IC) mice were inconsistent. The lack of deficit in the marble-burying protocol is also unexpected. The study by Malkova *et al.* found significantly increased marble burying in their male poly(IC) mice (2012). Xuan & Hampson found similar results for their male (but not female) mice, while Schwartzer *et al*. observed increased marble burying for both sexes (2014; 2013).

Increased grooming in the single-chamber paradigm of our study showed a marked abnormality for poly(IC) mice. They groomed significantly more than saline controls, indicating an autistic-like increase in self-directed repetitive behavior. Malkova *et al.* found similar results while Xuan & Hampson did not detect a deficit in this measure. Grooming behavior in the 3-chamber paradigm adds further question to the autistic phenotype of our poly(IC) mouse model. Grooming times of poly(IC) mice in the first and second phase of the 3-chamber paradigm is thus far poorly researched. Our data revealed significantly increased grooming in the social situation phase 2 for both saline and poly(IC) mice

compared to non-social situation phase 1. In female mice, the increase in grooming was far more pronounced in saline mice, which groomed significantly more than poly(IC) females in phase 2. Therefore, our data did not indicate that the poly(IC) had increased self-directed behavior in the social setting.

The difference seen in data between grooming in the 3-chamber test and in a single-chamber test may require further explanation. While both the phase 1 grooming and the single chamber grooming were examples of grooming time in a non-social setting, data did not look the same between the two measures. It should be noted that in the 3-chamber protocol, phase 1 grooming is measured during the first 10 minutes that the mouse has gained access to all three chambers of the apparatus. It is hypothesized that the resulting exploratory behavior diminishes grooming that is otherwise observed in the single chamber protocol (an environment to which the mouse has already been habituated 10 minutes prior to timing of stereotypy). Having already exhausted exploration during habituation, greater grooming levels are seen in the single chamber protocol allowing for differences to be detected.

Phase 2 grooming data provides different information than that given by the single chamber protocol because grooming during phase 2 of the 3-chamber test, the social setting, is often interpreted as an indicator of social anxiety. That is, a mouse is considered socially anxious if it grooms more during the social phase (Mines *et al.*, 2010; McNaughton *et al.*, 2008). Xuan & Hampson likewise found that while there was a trend towards increased grooming, neither male nor female poly(IC) mice groomed significantly more in the social phase of the 3-chamber test than saline controls (2014). While other literature regarding this particular behavioral measure could not be found, these two studies in

conjunction suggest that the poly(IC) mouse model may not exhibit an abnormality in this measure of social condition grooming.

Social Communication

In this study, social communication was tested using the social transmission of food preference task. Our results indicated that poly(IC) mice performed this task without deficit. To the author's knowledge this measure has not been used to study poly(IC) mice with the exception of a pilot study which discovered a similar lack of deficit (Murphy, 2014). This information contributes to the growing body of knowledge surrounding the behavioral phenotype of the poly(IC) mouse model of autism. Other studies that have assessed communication have looked at vocalizations. Schwartzer *et al*. found no abnormalities in the poly(IC) mice (2013). However, Malkova *et al*. (2012) found that poly(IC) pups, compared to controls, emit fewer ultrasonic vocalizations in response to a separation from their mother. Furthermore, the vocalizations are abnormal. In adults, Malkova *et al*. saw a communication deficit in male poly(IC) scent-marking in response to female urine.

Effects of the ketogenic diet on the poly(IC) mouse

Blood and Weight

Our study found significant impacts of KD on blood and weight measurements. As expected, three weeks on the 6.6:1 (carbohydrates: (fats+proteins)) strict KD resulted in significantly increased ketone levels and significantly decreased glucose levels. Furthermore, poly(IC) mice on the KD did not gain weight between 5 and 8 weeks of age and weighed significantly less than the control diet groups at 8 weeks of age. This is indicative of caloric or protein restriction for the KD group. The significant difference of

blood ketone levels seen between males and females is not unexpected, as higher ketone levels in females have been well documented (Deuel *et al*., 1937). The reason for the significant difference in ketone levels of male and female poly(IC) mice on the control diet is unclear. However, ketone levels are overall quite low in this treatment-diet group, so the difference is of uncertain biological importance.

BEHAVIOR

Despite the unexpected results regarding our poly(IC) mice, significant and notable effects of KD on the poly(IC) were observed. In males, the poly(IC)-KD group was significantly more social in the 3-chamber test, and for both sexes, sociability in the form of social contact was significantly increased. Grooming in the social phase 2 of the 3-chamber test was significantly lower than both other groups for the male poly(IC) mice and significantly lower than the saline group for female poly(IC) mice. Despite the sex difference, KD mice had lower grooming times overall in this measure. KD reversed poly(IC)-elevated grooming in the single chamber test. A diet effect was not seen in the marble burying task. The sex differences seen in the 3-chamber sociability and grooming may be a result of a less pronounced deficit in the poly(IC) mice as discussed above.

Overall, KD increased sociability and decreased grooming time, behavioral effects considered beneficial in light of the core symptoms of ASD. These results are in agreement with previous findings that have demonstrated that KD alleviates social deficits and increased stereotyped behaviors in mouse models of autism. Ruskin *et al.* (2013) found that a strict KD reduced grooming in the single chamber and 3-chamber test and increased preference for sociability and social contact in the 3-chamber test for the BTBR mouse model of autism. Another study investigating the effect of KD on EL mice, a mouse model of

epilepsy and autism, found that strict KD improved autistic symptoms for female EL mice, decreasing self-directed repetitive behavior and increasing sociability within the 3-chamber test (Fortin, 2014). The EL and BTBR mice are genetic in etiology while the poly(IC) is environmental. Together, these results indicate that KD may be a promising diet therapy for autism, both genetic and environmental in etiology.

The mechanisms by which KD may be beneficial are relatively unknown. However, it is suggested that saturated fatty acids, the levels of which are raised by the diet, activate toll-like receptors contribute to an anti-inflammatory effect (Forsythe *et al*., 2008). KD also fundamentally alters metabolism which is often abnormal in autistic patients (Chauan *et al.,* 2011). Furthermore, KD increases adenosine in the brain. The promotion and reduction of adenosine's function is associated with the alleviation and promotion several symptoms and comorbidities of autism, respectively (Masino *et al.*, 2009; Masino *et* al., 2011; Poon & Sawynok, 1999; Freitag *et al.,* 2009; Bottini *et al.*, 2001). Further research is needed to fully explore the mechanisms and applications of KD.

CONCLUSIONS AND FUTURE DIRECTIONS

This study demonstrated a clear influence of strict KD on behavior in the poly(IC) mice towards the improvement of sociability and alleviation of self-directed stereotyped behavior. While our study did not succeed in replicating several autistic behaviors in the poly(IC) mouse model, these effects of KD suggest that KD may be an effective diet therapy for autism and indicate a strong need for further research.

In the future, research regarding the effect of KD on the autistic symptoms of the poly(IC) mouse model may use altered methods to elicit a clear deficit in the poly(IC) mice and ensure a lack of abnormality in the control group. Strategies towards this goal may include utilizing pups from an un-injected dam for further controls. A single injection procedure rather than the three injection procedure used here may reduce maternal stress caused by repeated handling. To assess the degree of immune response from both saline and poly(IC) injected dams, inflammation markers indicative of immune response may be monitored. Additionally, the injection of interleukin-6 (IL6) instead of poly(IC) may be considered. This pro-inflammatory cytokine is a critical mediator of the effect of poly(IC) (Smith *et al.*, 2007). Direct injection of IL6 may reduce variability of behavior in offspring. Furthermore, while the sex differences seen in our data is not wholly unexpected and a group effect of estrus cycle should have been eliminated through the random testing of our procedure, monitoring estrus cycle in our female test mice may be illuminating.

REFERENCES

Abel, K. M., Dalman, C., Svensson, A. C., Susser, E., Dal, H., Idring, S., ... & Magnusson, C. (2013). Deviance in fetal growth and risk of autism spectrum disorder. *American Journal of Psychiatry*, *170*(4), 391-398.

Amaral, D. G., Schumann, C. M., & Nordahl, C. W. (2008). Neuroanatomy of autism. *Trends in Neurosciences*, *31*(3), 137-145.

American Psychiatric Association. APA (2013). *Diagnostic and Statistical Manual of Mental Disorders*, *5*.

Arrode-Brusés, G., & Brusés, J. L. (2012). Maternal immune activation by poly (I: C) induces expression of cytokines IL-1beta and IL-13, chemokine MCP-1 and colony stimulating factor VEGF in fetal mouse brain. *Journal of Neuroinflammation*, *9*(8), 1-16.

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I. N., & Van de Water, J. (2011). Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *Journal of Neuroimmunology*, *232*(1), 196-199.

Atladóttir, H. Ó., Thorsen, P., Østergaard, L., Schendel, D. E., Lemcke, S., Abdallah, M., & Parner, E. T. (2010). Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *Journal of Autism and Developmental Disorders*, *40*(12), 1423-1430.

Bauman, M. D., Iosif, A. M., Smith, S. E., Bregere, C., Amaral, D. G., & Patterson, P. H. (2014). Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological Psychiatry*, *75*(4), 332-341.

Boksa, P. (2010). Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain, behavior, and immunity*, *24*(6), 881-897.Atladóttir, H. Ó., Thorsen, P., Østergaard, L., Schendel, D. E., Lemcke, S., Abdallah, M., & Parner, E. T. (2010). Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *Journal of Autism and Developmental Disorders*, *40*(12), 1423-1430.

Bottini, N., De Luca, D., Saccucci, P., Fiumara, A., Elia, M., Porfirio, M. C., ... & Curatolo, P. (2001). Autism: evidence of association with adenosine deaminase genetic polymorphism. *Neurogenetics*, *3*(2), 111-113.

Careaga, M., Van de Water, J., & Ashwood, P. (2010). Immune dysfunction in autism: a pathway to treatment. *Neurotherapeutics*, *7*(3), 283-292.

Chadman, K. K. (2011). Fluoxetine but not risperidone increases sociability in the BTBR mouse model of autism. *Pharmacology Biochemistry and Behavior, 97*(3), 586-594.

Chauhan, A., Gu, F., Essa, M. M., Wegiel, J., Kaur, K., Brown, W. T., & Chauhan, V. (2011). Brain region-specific deficit in mitochondrial electron transport chain complexes in children with autism. *Journal of Neurochemistry*, *117*(2), 209-220.

Chez, M. G., Dowling, T., Patel, P. B., Khanna, P., & Kominsky, M. (2007). Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. *Pediatric Neurology*, *36*(6), 361-365.

Croen, L. A., Grether, J. K., Yoshida, C. K., Odouli, R., & Van de Water, J. (2005). Maternal autoimmune diseases, asthma and allergies, and childhood autism spectrum disorders: a case-control study. *Archives of Pediatrics & Adolescent Medicine*, *159*(2), 151-157.

Cullingford, T. E. (2004). The ketogenic diet; fatty acids, fatty acid-activated receptors and neurological disorders. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *70*(3), 253-264.

Currenti, S. A. (2010). Understanding and determining the etiology of autism. *Cellular and Molecular Neurobiology*, *30*(2), 161-171.

Czeizel, A. E. (2005). Birth defects are preventable. *International Journal of Medical Sciences*, *2*(3), 91.

Dean, J. C. S., Hailey, H., Moore, S. J., Lloyd, D. J., Turnpenny, P. D., & Little, J. (2002). Long term health and neurodevelopment in children exposed to antiepileptic drugs before birth. *Journal of Medical Genetics*, *39*(4), 251-259.

Deuel, H. J., Hallman, L. F., Murray, S., & Samuels, L. T. (1937). The sexual variation in carbohydrate metabolism viii. The rate of absorption of glucose and of glycogen formation in normal and adrenalectomized rats. *Journal of Biological Chemistry*, *119*(2), 607-615.

Engel, S. M., & Daniels, J. L. (2011). On the complex relationship between genes and environment in the etiology of autism. *Epidemiology*, *22*(4), 486-488.

Fein, D., Barton, M., Eigsti, I. M., Kelley, E., Naigles, L., Schultz, R. T., ... & Tyson, K. (2013). Optimal outcome in individuals with a history of autism. *Journal of Child Psychology and Psychiatry*, *54*(2), 195-205.

Forsythe, C. E., Phinney, S. D., Fernandez, M. L., Quann, E. E., Wood, R. J., Bibus, D. M., ... & Volek, J. S. (2008). Comparison of low fat and low carbohydrate diets on circulating fatty acid composition and markers of inflammation. *Lipids*, *43*(1), 65-77.

Freitag, C. M., Agelopoulos, K., Huy, E., Rothermundt, M., Krakowitzky, P., Meyer, J., ... & Hohoff, C. (2010). Adenosine A2A receptor gene (ADORA2A) variants may increase autistic symptoms and anxiety in autism spectrum disorder. *European Child & Adolescent Psychiatry*, *19*(1), 67-74.

Ganz, M. L. (2007). The lifetime distribution of the incremental societal costs of autism. *Archives of Pediatrics & Adolescent Medicine*, *161*(4), 343-349.

Gasior, M., Rogawski, M. A., & Hartman, A. L. (2006). Neuroprotective and disease-modifying effects of the ketogenic diet. *Behavioural Pharmacology*, *17*(5-6), 431.

Giulivi, C., Napoli, E., Schwartzer, J., Careaga, M., & Ashwood, P. (2013). Gestational exposure to a viral mimetic poly (I:C) results in long-lasting changes in mitochondrial function by leucocytes in the adult offspring. *Mediators of Inflammation*, *2013,* 1-8.

Grabrucker, A. M. (2012). Environmental factors in autism. *Frontiers in Psychiatry*, *3*, 1-8.

Hao, J., Liu, R., Turner, G., Shi, F. D., & Rho, J. M. (2012). Inflammation-mediated memory dysfunction and effects of a ketogenic diet in a murine model of multiple sclerosis. *PloS one*, *7*(5), e35476.

Helt, M., Kelley, E., Kinsbourne, M., Pandey, J., Boorstein, H., Herbert, M., & Fein, D. (2008). Can children with autism recover? If so, how?. *Neuropsychology Review*, *18*(4), 339-366.

Kim, D. Y., Davis, L. M., Sullivan, P. G., Maalouf, M., Simeone, T. A., Brederode, J. V., & Rho, J. M. (2007). Ketone bodies are protective against oxidative stress in neocortical neurons. *Journal of Neurochemistry*, *101*(5), 1316-1326.

Kim, S. H., & Lord, C. (2013). The behavioral manifestations of autism spectrum disorders. *The Neuroscience of Autism Spectrum Disorders*, 25-37.

Knapp, M., Romeo, R., & Beecham, J. (2009). Economic cost of autism in the UK. *Autism*, *13*(3), 317-336.

Laumonnier, F., Bonnet-Brilhault, F., Gomot, M., Blanc, R., David, A., Moizard, M. P., ... & Briault, S. (2004). X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *The American Journal of Human Genetics*, *74*(3), 552-557.

Lee, L. C., Harrington, R. A., Louie, B. B., & Newschaffer, C. J. (2008). Children with autism: Quality of life and parental concerns. *Journal of Autism and Developmental Disorders*, *38*(6), 1147-1160.

Li, X., Chauhan, A., Sheikh, A. M., Patil, S., Chauhan, V., Li, X. M., ... & Malik, M. (2009). Elevated immune response in the brain of autistic patients. *Journal of Neuroimmunology*, *207*(1), 111-116.

Lofthouse, N., Hendren, R., Hurt, E., Arnold, L. E., & Butter, E. (2012). A review of complementary and alternative treatments for autism spectrum disorders. *Autism Research and Treatment*, *2012*, 1-21.

Malkova, N. V., Collin, Z. Y., Hsiao, E. Y., Moore, M. J., & Patterson, P. H. (2012). Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain, Behavior, and Immunity*, *26*(4), 607-616.

Masino, S. A., Kawamura Jr, M., Wasser, C. D., Pomeroy, L. T., & Ruskin, D. N. (2009). Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. *Current Neuropharmacology*, *7*(3), 257.

Masino, S. A., Kawamura, M., Plotkin, L. M., Svedova, J., DiMario, F. J., & Eigsti, I. M. (2011). The relationship between the neuromodulator adenosine and behavioral symptoms of autism. *Neuroscience Letters*, *500*(1), 1-5.

Matson, J. L., Adams, H. L., Williams, L. W., & Rieske, R. D. (2013). Why are there so many unsubstantiated treatments in autism?. *Research in Autism Spectrum Disorders*, *7*(3), 466-474.

McNaughton, C. H., Moon, J., Strawderman, M. S., Maclean, K. N., Evans, J., & Strupp, B. J. (2008). Evidence for social anxiety and impaired social cognition in a mouse model of Fragile X syndrome. *Behavioral Neuroscience, 122*(2), 293–300.

Mines, M. A., Yuskaitis, C. J., King, M. K., Beurel, E., & Jope, R. S. (2010). GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of Fragile X syndrome and autism. *PLoS One, 5*(3), e9706.

Nadler, J. J., Moy, S. S., Dold, G., Simmons, N., Perez, A., Young, N. B., ... & Crawley, J. N. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes, Brain and Behavior, 3*(5), 303-314.

Nakazawa, M., Kodama, S., & Matsuo, T. (1983). Effects of ketogenic diet on electroconvulsive threshold and brain contents of adenosine nucleotides. *Brain and Development*, *5*(4), 375-380.

Naviaux, R. K., Zolkipli, Z., Wang, L., Nakayama, T., Naviaux, J. C., Le, T. P., ... & Powell, S. B. (2013). Antipurinergic therapy corrects the autism-like features in the poly (IC) mouse model. *PloS One*, *8*(3), e57380.

Ornoy, A. (2009). Valproic acid in pregnancy: how much are we endangering the embryo and fetus?. *Reproductive Toxicology*, *28*(1), 1-10.

Patterson, P. H. (2011). Maternal infection and immune involvement in autism. *Trends in Molecular Medicine*, *17*(7), 389-394.

Payne, N. E., Cross, J. H., Sander, J. W., & Sisodiya, S. M. (2011). The ketogenic and related diets in adolescents and adults—A review. *Epilepsia*, *52*(11), 1941-1948.

Persico, A. M., & Napolioni, V. (2013). Autism genetics. *Behavioural Brain Research*, *251*, 95-112.

Poon, A., & Sawynok, J. (1999). Antinociceptive and anti-inflammatory properties of an adenosine kinase inhibitor and an adenosine deaminase inhibitor. *European Journal of Pharmacology*, *384*(2), 123-138.

Rosenberg, R. E., Law, J. K., Yenokyan, G., McGready, J., Kaufmann, W. E., & Law, P. A. (2009). Characteristics and concordance of autism spectrum disorders among 277 twin pairs. *Archives of Pediatrics & Adolescent Medicine*, *163*(10), 907-914.

Rossignol, D. A., & Frye, R. E. (2012). Mitochondrial dysfunction in autism spectrum disorders: a systematic review and meta-analysis. *Molecular Psychiatry*, *17*(3), 290-314.

Ruskin, D. N., Kawamura Jr, M., & Masino, S. A. (2009). Reduced pain and inflammation in juvenile and adult rats fed a ketogenic diet. *PLoS One*, *4*(12), e8349.

Ruskin, D. N., Svedova, J., Cote, J. L., Sandau, U., Rho, J. M., Kawamura Jr, M., ... & Masino, S. A. (2013). Ketogenic diet improves core symptoms of autism in BTBR mice. *PloS One*, *8*(6), e65021.

Schmidt, R. J., Hansen, R. L., Hartiala, J., Allayee, H., Schmidt, L. C., Tancredi, D. J., ... & Hertz-Picciotto, I. (2011). Prenatal vitamins, one-carbon metabolism gene variants, and risk for autism. *Epidemiology (Cambridge, Mass.)*, *22*(4), 476.

Schwartzer, J. J., Careaga, M., Onore, C. E., Rushakoff, J. A., Berman, R. F., & Ashwood, P. (2013). Maternal immune activation and strain specific interactions in the development of autism-like behaviors in mice. *Translational Psychiatry, 3*(3), e240.

Shi, L., Fatemi, S. H., Sidwell, R. W., & Patterson, P. H. (2003). Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *The Journal of Neuroscience, 23*(1), 297-302.

Silverman, J. L., Smith, D. G., Rizzo, S. J. S., Karras, M. N., Turner, S. M., Tolu, S. S., ... & Crawley, J. N. (2012). Negative allosteric modulation of the mGluR5 receptor reduces repetitive behaviors and rescues social deficits in mouse models of autism. *Science Translational Medicine, 4*(131), 131ra51-131ra51.

Smith, S. E., Li, J., Garbett, K., Mirnics, K., & Patterson, P. H. (2007). Maternal immune activation alters fetal brain development through interleukin-6. *The Journal of Neuroscience, 27*(40), 10695-10702.

Stafstrom, C. E., & Rho, J. M. (2012). The ketogenic diet as a treatment paradigm for diverse neurological disorders. *Frontiers in Pharmacology*, *3*.

Stanfield, A. C., McIntosh, A. M., Spencer, M. D., Philip, R., Gaur, S., & Lawrie, S. M. (2008). Towards a neuroanatomy of autism: a systematic review and meta-analysis of structural magnetic resonance imaging studies. *European Psychiatry*, *23*(4), 289-299.

Sumi, S., Taniai, H., Miyachi, T., & Tanemura, M. (2006). Sibling risk of pervasive developmental disorder estimated by means of an epidemiologic survey in Nagoya, Japan. *Journal of Human Genetics*, *51*(6), 518-522.

Traynor, T. R., Majde, J. A., Bohnet, S. G., & Krueger, J. M. (2004). Intratracheal double-stranded RNA plus interferon-γ: a model for analysis of the acute phase response to respiratory viral infections. *Life Sciences*, *74*(20), 2563-2576.

Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W., & Pardo, C. A. (2005). Neuroglial activation and neuroinflammation in the brain of patients with autism. *Annals of Neurology*, *57*(1), 67-81.

Wingate, M., Kirby, R. S., Pettygrove, S., Cunniff, C., Schulz, E., Ghosh, T., ... & Yeargin-Allsopp, M. (2014). Prevalence of autism spectrum disorder among children aged 8 years-autism and developmental disabilities monitoring network, 11 sites, United States, 2010. *MMWR Surveillance Summaries*, *63*(2).

Xuan, I. C., & Hampson, D. R. (2014). Gender-Dependent Effects of Maternal Immune Activation on the Behavior of Mouse Offspring. *PloS One, 9*(8), e104433.

Yang, X., & Cheng, B. (2010). Neuroprotective and anti-inflammatory activities of ketogenic diet on MPTP-induced neurotoxicity. *Journal of Molecular Neuroscience*, *42*(2), 145-153.