ASSOCIATIONS BETWEEN PSYCHOPHYSIOLOGICAL, IMMUNE, ENDOCRINE, AND SEROTONERGIC BIOMARKERS OF STRESS AND GASTROINTESTINAL SYMPTOMS IN AUTISM SPECTRUM DISORDER

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ASSOCIATIONS BETWEEN PSYCHOPHYSIOLOGICAL, IMMUNE, ENDOCRINE, AND SEROTONERGIC BIOMARKERS OF STRESS AND GASTROINTESTINAL SYMPTOMS IN AUTISM SPECTRUM DISORDER

presented by Bradley James Ferguson,

a candidate for the degree of doctor of philosophy in neuroscience, and hereby certify that, in their opinion, it is worthy of acceptance.

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To my wife, Heather Ferguson, and my parents, Bill and Kathy Ferguson, for their enduring support, love, and patience over the years. To the countless individuals along this journey who provided me with encouragement and support. Thank you.

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LIST OF ABBREVIATIONS

ABC	Aberrant Behavior Checklist
ADOS	Autism Diagnostic Observation Schedule
ANS	Autonomic Nervous System
ANOVA	Analysis of Variance
ASD	Autism Spectrum Disorder
DSM	Diagnostic and Statistical Manual of Mental Disorders
FC	Functional Constipation
FFQ	Food Frequency Questionnaire
FSIQ	Full-scale Intelligence Quotient
GI	Gastrointestinal
HRV	Heart Rate Variability
IL-6	Interleukin-6
IQ	Intelligence Quotient
MS	milliseconds
MU	University of Missouri
pNN50	Percentage of Successive Normal R-R Intervals Differing by 50ms or More
QPGS	Questionnaire of Pediatric Gastrointestinal Symptoms – Rome III
RBS-R	Repetitive Behavior Scale – Revised
SCL	Skin Conductance Level
SD	Standard Deviation
STAI	State-Trait Anxiety Inventory
TNF-α	Tumor necrosis factor-alpha

ASSOCIATIONS BETWEEN ENDOCRINE, IMMUNE, SEROTONERGIC, AND PSYCHOPHYSIOLOGICAL BIOMARKERS OF STRESS AND GASTROINTESTINAL SYMPTOMS IN AUTISM SPECTRUM DISORDER

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ABSTRACT

Autism spectrum disorder (ASD) is often accompanied by gastrointestinal (GI) disturbances, which also may impact behavior. Alterations in autonomic, endocrine, and immune system functioning are also frequently observed in ASD, however, the relationship between these findings in ASD is not known. To study this relationship, an initial pilot study was conducted in our lab which revealed increased autonomic nervous system (ANS) functioning in response to brief stress-invoking peripheral stimuli and GI disorders in ASD. Preliminary data suggested an enhanced stress response in individuals with ASD and co-occurring GI disorders. In a subsequent multi-site study, we examined the relationship between GI symptomatology, examining upper and lower GI tract symptomatology separately, ANS functioning, and salivary cortisol at baseline and in response to stress in a sample of 120 children with ASD. The stress-associated pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) and whole blood serotonin concentrations were also assessed. While the number of participants with significant upper GI tract problems was small in this sample, 42.5% of participants met criteria for functional constipation, a disorder of the lower GI tract. Heart rate variability, a measure of parasympathetic modulation of cardiac activity, was found

to be positively associated with lower GI tract symptomatology at baseline. This relationship was particularly strong for participants with co-occurring diagnoses of anxiety disorder and for those with a history of regressive ASD or loss of previously acquired skills. A greater amount of lower GI tract symptoms was significantly associated with post-stress cortisol concentration, and this relationship was greatest for individuals with regressive ASD. However, symptoms of the lower GI tract were not associated with the stress-responsive cytokines IL-6 and TNF-α. Finally, in a sample of 82 of the 120 children mentioned above, a significant positive correlation was found between lower GI tract symptoms and whole-blood serotonin. These findings suggest that systems involved in the response to mild stimuli are different in individuals with ASD and co-occurring GI issues, especially for constipation; although it is not possible to assess causality in this data set. Future work should examine the impact of treatment of GI problems on autonomic function and anxiety, as well as the impact of anxiety treatment on GI problems. Thus, clinicians should be aware that GI problems, anxiety, autonomic, endocrine, and immune dysfunction may cluster in children with ASD and should be addressed in a multidisciplinary treatment plan.

CHAPTER ONE:

INTRODUCTION

In 1908, Swiss psychiatrist Eugen Bleuler coined the term 'autism,' where it was first used to describe a patient with schizophrenia who was "withdrawn" and "self-absorbed." The Greek word "autos" translates to "self," and so the word "autism" was used to reflect this as "morbid self-admiration and withdrawal within the self." It would not be until 1943 that the word autism was used in a modern sense, where psychiatrist Leo Kanner first described in his seminal paper in *Nervous Child* titled, "Autistic disturbances of affective contact," 11 children – 8 boys and 3 girls – "whose condition differs so markedly and uniquely from anything reported so far, that each case merits – and I hope, will eventually receive – a detailed consideration of its fascinating peculiarities." (Kanner, 1943). Some accounts include children who "developed mania for spinning blocks and pans and other round objects," and complaints of "adaptive behavior in a social setting characterized by attacking as well as withdrawing behavior." Some of the children were mute or had language deficits and echolalia, - the repetition of verbal messages that were previously named and heard – while others had excellent rote memory and superior intellect. Many of the children described by Kanner also had difficulty with feeding, and characterized by regurgitation and vomiting. Then, in 1944, the Austrian pediatrician Hans Asperger described a pattern of behavior and abilities that he called "autistic psychopathy," meaning autism (self) and psychopathy (personality). This pattern of personality traits included individuals with "a lack of empathy, little ability to form friendships, one-sided conversation, intense absorption in a special interest, and clumsy movements." Asperger even referred to these children as "little

professors," given their ability to speak about their favorite subject in extreme detail. Over the next several decades after the accounts from Bleuler, Kanner, and Asperger, the definition of autism would be changed several times in the *Diagnostic and Statistical Manual for Mental Disorders*. Today, the *Diagnostic and Statistical Manual for Mental Disorders – V* defines autism spectrum disorder (ASD) as "a disorder of neurodevelopment, characterized by persistent deficits in social communication and interaction and restricted, repetitive patterns of behavior that occur early in development" (American Psychiatric Association, 2013). The most current prevalence estimate for ASD is 1:68 – that is, 1 out of every 68 children born in the United States will be diagnosed with ASD (Centers for Disease Control and Prevention, 2014). The disorder disproportionately affects males at a ratio of 4:1.

Research suggests that the prevalence of gastrointestinal (GI) disorders is elevated in ASD when compared to individuals without ASD. Studies suggest an augmented stress response is characteristic of some individuals with ASD, and a heightened stress response is associated with GI dysfunction in those without ASD. Thus, the following program of research presented in this dissertation examines relationships between different systems in the body which are altered by the stress response and GI symptoms in individuals with ASD. Increasing our knowledge of the potential relationship between the stress response and GI symptoms in ASD may lead to the discovery of treatment biomarkers, a necessary component in the development of precision medicine (Beversdorf, 2016).

Gastrointestinal Disorders in ASD

Co-occurring medical conditions, such as GI disorders, are common in ASD, though reports vary on the prevalence of GI disorders relative to typically-developing

children (McElhanon, McCracken, Karpen, & Sharp, 2014; Doshi-Velez, Ge, & Kohane, 2014; Chaidez, Hansen, & Hertz-Picciotto, 2014; Chandler et al., 2013; Gorrindo et al., 2012; Bauman, 2010; Buie et al., 2010; Mouridsen, Rich, & Isager, 2010; Ibrahim, Voigt, Katusic, Weaver, & Barbaresi, 2009). Recent data suggests that GI symptomatology arises early in the course of ASD (Bresnahan et al., 2015). Many individuals with ASD are non-verbal and are unable to report painful GI symptoms. As such, non-GI problem behavior may serve as a marker of abdominal pain and discomfort in ASD (Buie et al., 2010). For instance, irritability, sleep disturbance, and aggression have been shown to be significantly increased in those with ASD relative to their typically developing siblings (Hovarth & Perman, 2002[a,b]). Furthermore, children with ASD and regression/loss of previously acquired skills have been shown to have a higher frequency of GI symptoms when compared to those with ASD without regression (Valicenti-McDermott, McVicar, Cohen, Wershil, & Shinnar, 2008). Despite these significant behavioral patterns, the pathophysiology associated with GI problems in ASD is poorly understood.

Autonomic Nervous System Functioning in ASD

Many psychophysiological studies suggest that individuals with ASD have altered autonomic nervous system (ANS) functioning relative to typically developing controls. For example, studies have shown increased sympathetic tone in individuals with ASD relative to typically-developing controls by measuring skin sweat, termed electrodermal activity (EDA), at baseline (van Engeland, 1984; Hirstein, Iversen, & Ramachandran, 2001) and in relation to various types of stimuli (Barry & James, 1988; Joseph, Ehrman, & McNally, 2008; Kylliäinen & Hietanen, 2006; Toichi & Kamio, 2003). Studies have found similar results upon examining cardiac measures of the

sympathetic/parasympathetic balance indicating reduced cardiac vagal tone in ASD relative to typically-developing controls (Ming, JuLu, Brimacombe, Conner, & Daniels, 2005; Toichi & Kamio, 2003). The results of these studies provide a body of evidence to suggest an enhanced response to stress exists in many individuals with ASD.

Cytokine, Cortisol, and Serotonergic Alterations in ASD

Activation of the HPA axis by stress results in immune system responses throughout the body, and individuals with ASD have been found to have an atypical immune response. Atypical markers in ASD that have been reported include alterations in IL-12, IFN-γ, IL-2, IL-6, IL-10, and TNF-α (Dinan et al., 2006). For TNF-α, IL-12, IL-10, IL-6, and IL-1β, the abnormalities have been associated with GI symptomatology (Dinan et al., 2006; Aggarwal et al., 1994). Immune alterations have also been associated with behavioral symptoms in ASD (Mayer, 2000). Furthermore, there is a significant overlap between what is observed in the autism population and the immune markers most associated with the stress response, especially for the pro-inflammatory cytokines IL-6 and TNF-α (Ming et al., 2005; Spratt et al., 2012).

Exposure to a social situation is associated with an enhanced cortisol response in ASD relative to typically- developing peers (Corbett et al., 2010), and there is a positive relationship between cortisol and self-reported social stress and anxiety during social situations in ASD (Lopata et al., 2008). Furthermore, a heightened cortisol response is linked to decreased intelligence as well as receptive and expressive language (Kidd et al., 2012), and some have proposed that the effects of stress on the HPA axis may contribute to these outcomes (Maldonado et al., 2008).

The serotonergic system is intimately involved with the stress response from numerous lines of evidence, including genetics, psychopharmacological, and neuroimaging findings (Peters et al., 2014). Whole blood serotonin has also long been known to be elevated in patients with ASD (Lopara et al., 2008). Whole blood serotonin is also elevated after experimentally induced stress (Kidd et al., 2012; Maldonado et al., 2008), and increased whole blood serotonin is associated with greater stress reactivity in the general population (Wei et al., 2012). Furthermore, recent data has convincingly demonstrated an association between ASD and heterogeneity at the SLC6A4 locus of the serotonin transporter, further supporting a critical role of the serotonergic system in ASD (Ashwood et al., 2011). Synthesis of blood 5-HT is performed in the gut where it is associated with inflammation and gut motility, making it an interesting biomarker for GI dysfunction in ASD (Gershon 2013). Also, 5-HT has been shown to mediate immunoregulation, which is important in the etiology in ASD (Jaiswal et al., 2015). Interestingly, a recent report found 5-HT levels to be associated with the stress-responsive pro-inflammatory cytokine IL-6, which is associated with ASD severity (Yang et al., 2015), suggesting an interaction between 5-HT and immune system functioning. Therefore, examination of whole blood serotonin would be of particular interest in how it interacts with stress, immune functioning, and gastrointestinal disorders in autism spectrum disorders.

Associations Between Stress and GI Functioning

Recent data suggests that the brain and gut communicate with each other in a bidirectional manner through the central, autonomic, and enteric nervous systems (Collins, Surette, & Bercik, 2012; Mayer, 2011; Scott, Clarke, & Dinan, 2013). The

vagus nerve, a component of the parasympathetic branch of the autonomic nervous system (ANS), couples the gut to the nucleus of the solitary tract in the brain stem, and is the primary afferent pathway from the abdomen to the brain (Gillis, Quest, Pagini, & Norman, 1989). Postganglionic sympathetic efferents project to the gut from the spinal cord, and synapse on the myenteric plexus to inhibit GI function (Aziz & Thompson, 1998). Thus, investigating the relationship between ANS function and GI symptomatology in ASD appears to be an important priority.

In the general population, there is a strong relationship between psychological and physical stress and GI disorders, and this may interact directly with gut bacteria to increase bacterial growth and infectivity (Lyte, Vulchanova, & Brown, 2011). Stress activates the hypothalamic-pituitary-adrenal axis, resulting in the neuronal release of catecholamines, activating the sympathetic nervous system (Elenkov & Chrousos, 2006), which has been shown to affect the gut mucosa (Lyte, et al., 2003). Sympathetic efferents can inhibit gut motility (Lomax, Harkey, & Furness, 2010; Hirst & McKirdy, 1974), suggesting a mechanism for constipation. As previously mentioned, this may involve bidirectional communication between the enteric nervous system, the intrinsic, reflexive nervous system of the GI tract, and the central nervous system. Increased sympathetic functioning and decreased parasympathetic functioning have both been noted in individuals with constipation predominant irritable bowel syndrome (IBS) (Mazur Fugala, Jablonski, Mach, & Thor, 2012), in association with a range of autonomic disturbances in IBS (Martinez-Martinez, Mora, Vargas, Fuentes-Iniestra, & Martinez-Lavin, 2014; Pellissier, Dantzer, Canini, Mathieu, & Bonaz, 2010), though this literature is still evolving (Mazurak, Seredyuk, Sauer, Teufel, & Enck, 2012). Diarrhea can also be

frequently observed, however, as part of the stress reaction, which includes sympathetic activation, in patients with irritable bowel syndrome (Bouchoucha, Hejnar, Devroede, Babba, & Benamouzig, 2013). Levels of the pro-inflammatory cytokines IL-6 and TNF-α are associated with GI dysfunction in individuals without ASD, suggesting a complex interaction between the immune system and GI dysfunction which may be relevant to ASD. Despite the literature describing alterations in both ANS and GI function in ASD, little is known about the relationship between these two systems in ASD.

Rationale for the Forthcoming Studies

Research has shown that the stress response is associated with GI dysfunction in the general population. Furthermore, many individuals with ASD have significant GI dysfunction, especially constipation, as well as an enhanced response to stress. One of the most common treatments for constipation in ASD is propylene glycol, however, not all individuals with ASD benefit from this treatment, suggesting the need for alternate treatments. The first study in this dissertation was a pilot study to explore relationships between ANS functioning at rest and in response to mildly stressful peripheral sensory stimulation in a small group of individuals with ASD (Chapter 2). Based on the findings of the pilot study, a multisite study involving 120 children was conducted to further examine the relationship between GI symptoms and ANS functioning in response to stress (Chapter 3), as well as the endocrine response to stress and pro-inflammatory immune system biomarkers and their associations with GI symptoms (Chapter 4). Furthermore, levels of whole blood serotonin (Chapter 5) were examined in this same group of children. Given the findings from the aforementioned studies, a possible mechanism for constipation in some cases with ASD is proposed at the conclusion of this

dissertation along with the discussion of a possible treatment for individuals with ASD and constipation that do not respond to propylene glycol. The reader should be aware that Chapters 3-5 were published in peer-reviewed journals prior to the completion of this dissertation, thus, some sections of this dissertation are redundant in an attempt to maintain the integrity of the articles.

CHAPTER TWO:

ASSOCIATIONS BETWEEN SKIN CONDUCTANCE LEVEL AND GASTROINTESTINAL SYMPTOMS IN ASD: A PILOT STUDY

Introduction

Many studies suggest the presence of an elevated incidence of GI disorders in children with autism. However, the incidence varies widely across reports (Buie et al, 2010). Regardless, gastrointestinal disorders are important due to their potential to exacerbate problem behaviors in ASD (Buie et al, 2010). Due to this relationship, there has been some speculation that the GI system might somehow contribute to the etiology of autism. However, firm evidence for this is lacking (Buie et al., 2010). It is well known, though, in the general population that there is a strong relationship between stress and GI disorders (Lyte, Vulchanova, & Brown, 2011). Additionally, evidence suggests an augmented stress response in autism (Corbett et al, 2008). Interestingly, recent evidence has shown that norepinephrine has a significant effect on the interaction between mucosal protection and the bacteria that populate the mucosal surface, suggesting a mechanism of action for the effect of stress on the GI system (Lyte, Vulchanova, & Brown, 2011). Therefore, understanding the relationship between stress reactivity and GI problems, including markers of sympathetic tone, in ASD seems critical to understanding the nature of ASD-related GI symptomatology. Furthermore, identifying the aspects contributing to GI problems in autism will be important for optimizing treatment strategies.

To begin to address this line of questions, a pilot study was performed. Due to the well-known relationship between stress and GI disorders in the general population, and evidence suggesting altered stress reactivity in ASD, we predicted altered autonomic

nervous system (ANS) functioning at rest and in response to brief stress-inducing peripheral stimulation among ASD patients with significant GI symptomatology as compared to those without such symptoms.

Methods

Participants. A sample of 9 patients diagnosed with ASD and a diagnosed GI disorder or significant GI symptomatology were compared to 8 age matched patients diagnosed with ASD with no GI symptoms (mean age = 10.63, *SD* = 4.4, range = 5-19). ASD diagnoses were confirmed by the Diagnostic Algorithm of the Autism Diagnostic Interview-Revised (ADI-R) (Lord, Rutter, & Couteur, 1994). ADI-R scores were obtained from existing clinical records at the University of Missouri (MU) Thompson Center for Autism & Neurodevelopmental Disorders in Columbia, Missouri. The study was approved by the MU Health Sciences Institutional Research Board, and all participants provided consent or assent where appropriate.

Gastrointestinal Symptoms. GI symptoms were assessed by parental report on the Questionnaire on Pediatric Gastrointestinal Symptoms, Rome III (QPGS). The QPGS questionnaire was scored in accordance with the scoring algorithm included with the questionnaire. Participants meeting Rome III criteria for any GI disorder per the QPGS were included in the ASD GI group, while participants not meeting Rome III criteria were included in the ASD NO GI group.

Skin Conductance Level. To obtain SCL data, two TSD203 Ag-AgCl skin resistance transducers (BIOPAC Systems, Goleta CA) filled with isotonic electrode gel were affixed to the distal phalanges of the index and middle fingers of each participant. The TSD203 transducers were connected to a BIOPAC MP150 Data Acquisition System (BIOPAC

Systems, Goleta, CA) and amplified using a GSR100C (BIOPAC Systems, Goleta, CA). The following amplifier settings were used on the GSR100C: Gain = 5μʊ/V; Low pass filter = 1.0Hz; High pass filters = both set to DC. A laptop computer with Acqknowledge version 4.1 software (BIOPAC Systems, Goleta, CA) was used to view the SCL data in real time. The data were saved for offline processing. During data processing, the SCL data were visually inspected for artifacts, and any records with excessive artifacts were not included in the final analysis. The resulting SCL data for each condition, as described below, was represented by the average SCL in microsiemens (μS) for the duration of the stress condition, which has been established in the literature as an index of sympathetic activity (Dawson, Schell, & Filion, 2007).

Electrocardiogram. To obtain electrocardiogram (ECG) data, one lead was placed below the right clavicle, in the mid-clavicular line within the frame of the rib cage, and the other on the lower left abdomen within the rib cage frame. A ground was obtained through the VIn- connection on the EDA100C amplifier, and so it follows that a grounding ECG lead was not placed on the chest. After placing electrodes on the participant, the ECG signal was verified by observing a series of characteristic QRS complexes. Data were collected at 1kHz, and the ECG-100C amplifier settings were as follows: Gain = 1000; R-wave mode: NORM; 35Hz LPN: ON; HP: 0.5Hz. The same laptop computer mentioned above was used to monitor the ECG signal in real time, and the resulting data were saved to the laptop for offline processing. The ECG data were processed by averaging the R-R intervals during each condition using AcqKnowledge version 4.1 software (BIOPAC Systems, Inc., Goleta, CA). Thus, the mean R-R interval,

or the amount of time between R peaks, represented the dependent variable for each condition.

Stress Reactivity Protocol. Participants were seated in a chair at a table with the researcher seated either in front of the participant or beside the participant. Upon attaching the finger transducers to the participant, as described above, the researcher informed the participant that they were to sit still and breathe normally for a period of 5 minutes. The purpose of this condition was to establish the participant's baseline reactivity to the testing environment. Next, the participant was informed that they were going to listen to some sounds (auditory condition), feel some vibrations with their hands (vibrotactile condition), and put their hands in cold water (coldpressor condition). For the vibrotactile and cold pressor conditions, stimuli were applied to each hand independently. The researcher administered the aforementioned stimuli in a counterbalanced fashion in order to control for the possibility that one stimulus condition may influence subsequent stimulus conditions. Furthermore, upon completion of each stimulus condition, a wash out period of 3 minutes was utilized before beginning the next condition, where to participant sat quietly in their chair. For the vibrotactile and coldpressor conditions, a washout period was also used after the application of stimuli to each hand. For the vibrotactile condition, this washout period was also implemented between the application of 'low' and 'high' speed stimulations to each hand.

For the auditory stimulus condition, participants wore a pair of closed ear headphones, and a fire engine siren was played at 90 dB for 10 second intervals with either 15 or 19 seconds of rest between each interval to in an attempt to control for

expectancy effects. The total length of the auditory condition was 4 minutes and 8 seconds.

For the vibrotactile condition, participants were instructed to grasp a vibrating back massager (Conair WM200X, Stamford, CT), allowing their palm to touch the flat surface of the stimulator. Participants grasped the stimulator for 30 seconds, and trials were conducted with each hand, as described above. To test for effects of vibrotactile intensity, independent conditions of 'low' and 'high' speed conditions were examined. Vibrotactile stimulation to the hands has been shown to elicit changes in cardiovascular activity (Foster et al., 2013).

For the cold pressor condition, a cooler of ice water was prepared prior to the study session and was calibrated to 4°C. Participants were instructed to place their hand in the water up to their wrist for 30 seconds. After a 3-minute washout period, participants placed their other hand in the cold water, immediately followed by another 3-minute wash out period. The cold pressor test has been established in the research literature as a method for eliciting a momentary increase in sympathetic nervous activity, (Zvan, Zaletel, Pretnar, Pogacnik, & Kiauta, 1998).

Results

For the SCL data, an initial omnibus one-way ANOVA across the vibrotactile and cold pressor stimulus conditions, including baseline exposure to the testing environment, indicated that mean SCL activity was significantly greater for the ASD GI group when compared to the ASD NO GI group, $F(1,120) = 4.88$, $p = 0.03$. Furthermore, SCL was significantly greater for the right-handed low-frequency vibrotactile condition, $F = 1.67$,

$p = 0.049$. A trend toward significance was revealed for the left-handed high-frequency condition, $F = 3.28$, $p = 0.092$ (Figure 2.1.)

For the ECG data, an initial omnibus one-way ANOVA across the vibrotactile and cold pressor stimulus conditions, including baseline exposure to the testing environment, indicated that the mean R-R interval was significantly lower for the ASD GI group when compared to the ASD NO GI group, $F(1,107) = 21.95$, $p < 0.0001$. Next, to examine the effects of unilateral stimulation, separate ANOVAs were conducted for left- and right-handed vibrotactile and cold pressor stimulation. The mean R-R interval was significantly lower for the left-handed low-frequency vibrotactile condition for the ASD GI group when compared to the ASD NO GI group, $F=4.72$, $p = 0.047$. The mean R-R interval was significantly lower for the right-handed coldpressor condition for the ASD GI group when compared to the ASD NO GI group, $F = 3.21$, $p = 0.049$. Trends toward significance were noted for the right-handed low-frequency vibrotactile stimulation task ($F = 3.21$, $p = 0{,}093$) and the right-handed high-frequency vibrotactile condition ($F = 3.17$, $p = 0.095$ (Figure 2.2).

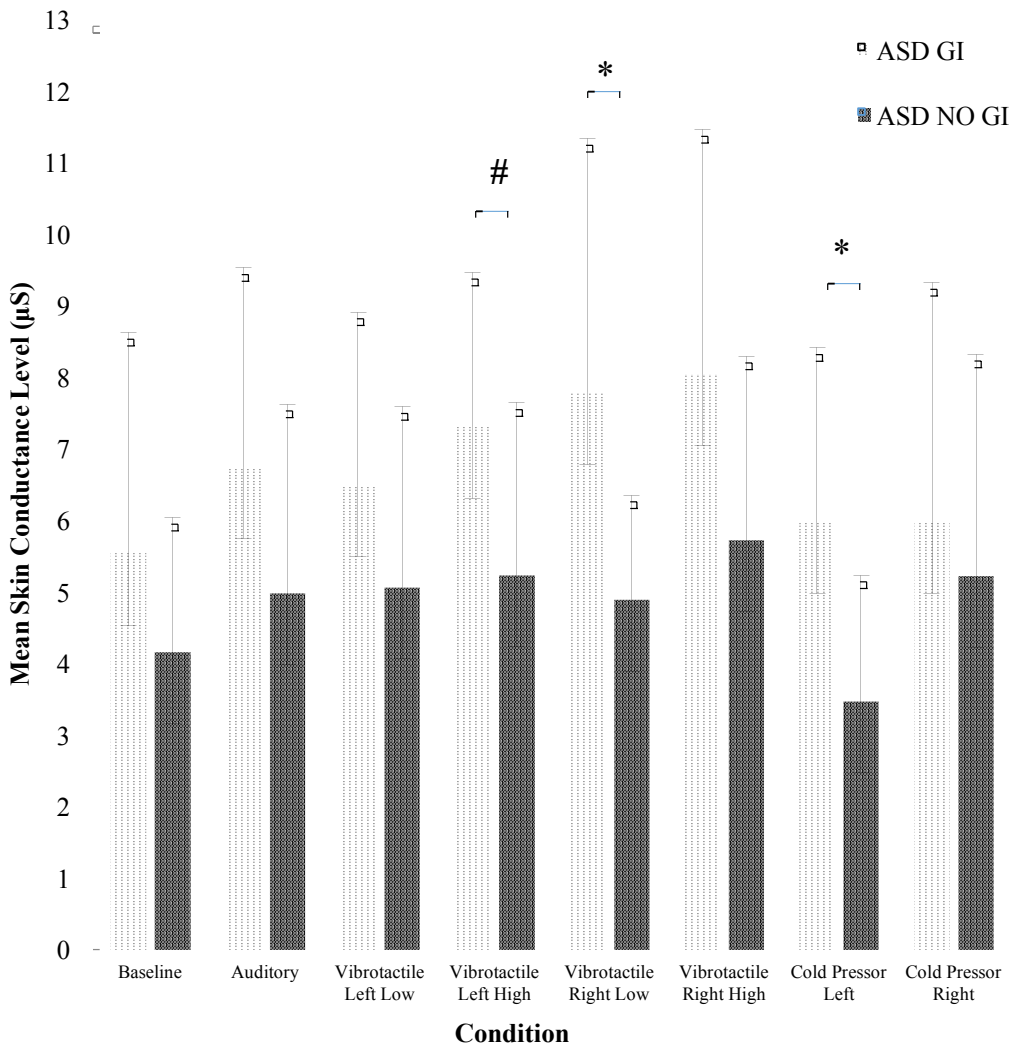

Figure 2.1. Mean GSR for Baseline, Auditory, Vibrotactile, and Cold Pressor Conditions. Bars indicate standard deviations of the mean. $^{*}p < 0.05$; $^{\#}p < 0.1$

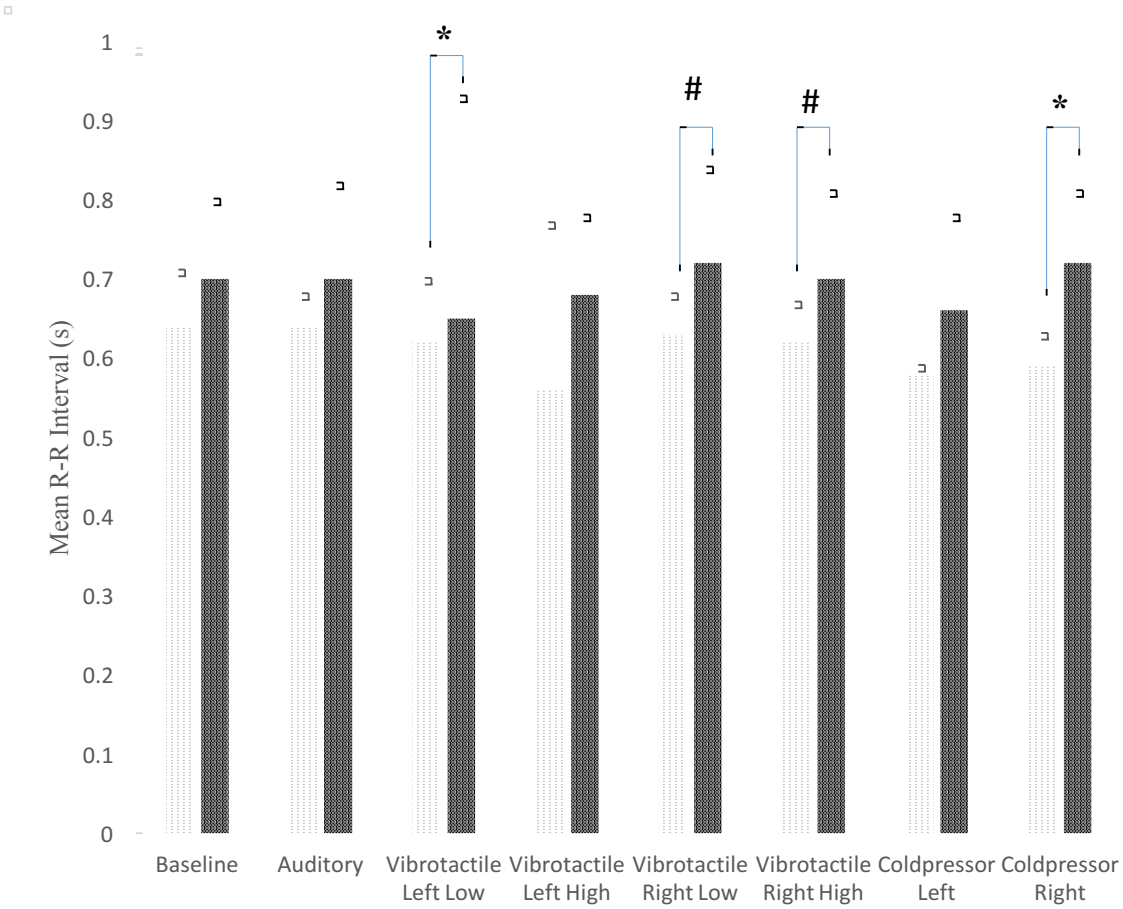

Figure 2.2. Mean R-R interval in seconds for ASD GI and ASD NO GI groups for each condition. Bars indicate standard deviations of the mean. $*p < 0.05$; $^{\#}p < 0.1$

Discussion

The results from this pilot study suggested that the psychophysiological response to sensory stimulation may differ in those with ASD and co-occurring GI symptomatology when compared to those with ASD alone. Specifically, an analysis including baseline, vibrotactile, and coldpressor stimulation indicated heightened SCL and reduced R-R interval in those with ASD and co-occurring GI issues compared to those with ASD alone. These findings provide preliminary evidence to suggest altered

ANS functioning in those with ASD and co-occurring GI issues, as SCL is a pure measure of sympathetic tone, and the R-R interval is of the balance of sympathetic and parasympathetic influences on the myocardium. Assessment of GI symptomatology in ASD is challenging, given that many children with ASD are nonverbal and are unable to report symptoms to their caregivers. Further, assessment of GI symptomatology occurs within the context of a clinical visit, and while the data obtained in this setting may be adequate for treatment purposes, it leaves much to be desired for the purposes of research. This is likely the reason that the prevalence of GI disorders varies from 10% to 90% (Buie et al., 2010). The present pilot study utilized the QPGS, which has been shown to be a valid and reliable measure of assessing GI symptomatology in children and adolescents. While the data obtained from this measure is categorical in nature, future studies utilizing the measure may further increase our knowledge of the range of GI symptomatology by creating a scoring rubric to allow the assessment of GI functioning in relation to stress-associated biomarkers on a continuous scale.

Although the present findings indicate group differences in the psychophysiological response to mildly stressful sensory stimulation, there are limitations. The sample size included a total of 17 individuals which limits the interpretation of the findings, and so future studies should aim to collect data from a large and diverse group of individuals with ASD. The present study utilized groupings based on Rome III criteria, however, the GI-stress relationship may vary on a continuum. Future studies would benefit from creating continuous scores of GI symptomatology while continuing to use a standardized questionnaire of GI symptoms such as the QPGS. As this study was conducted to obtain pilot data on the psychophysiological response to

peripheral stress in ASD, it may not be feasible to use with all individuals with ASD given the task demands. Thus, future studies may wish to examine other biomarkers associated with the stress response such as salivary cortisol, stress-associated inflammatory markers, and neurotransmitters such as serotonin. Obtaining biomarkers associated with GI disorders in individuals with ASD will further our knowledge of the GI-stress relationship which may lead to optimized treatment strategies in the future.

CHAPTER THREE:

PSYCHOPHYSIOLOGICAL ASSOCIATIONS WITH GASTROINTESTINAL SYMPTOMATOLOGY IN ASD[1]

Introduction

Recent data suggests that the brain and gut communicate with each other in a bidirectional manner through the central, autonomic, and enteric nervous systems (Collins, Surette, & Bercik, 2012; Mayer, 2011; Scott, Clarke, & Dinan, 2013). The vagus nerve, a component of the parasympathetic branch of the autonomic nervous system (ANS), couples the gut to the nucleus of the solitary tract in the brain stem, and is the primary afferent pathway from the abdomen to the brain (Gillis, Quest, Pagini, & Norman, 1989). Postganglionic sympathetic efferents project to the gut from the spinal cord, and synapse on the myenteric plexus to inhibit GI function (Aziz & Thompson, 1998). Thus, investigating the relationship between ANS function and GI symptomatology in ASD appears to be an important priority. Many psychophysiological studies suggest that individuals with ASD have altered ANS functioning relative to typically developing controls. Electrodermal activity (EDA), defined as the electrical conductivity between two electrodes on the skin over time, provides an index of sympathetic nervous system activity, since eccrine sweat glands are innervated by the sympathetic but not parasympathetic branch of the ANS (Boucsein, 2012). Studies have shown increased EDA in those with ASD relative to controls at baseline (van Engeland, 1984; Hirstein, Iversen, & Ramachandran, 2001), in response to visual and auditory stimuli (Barry & James, 1988), in response to facial stimuli (Joseph, Ehrman, & McNally, 2008; Kylliäinen & Hietanen, 2006), and in response to repetitive stimuli over

[1] Ferguson, B.J., Marler, S., Altstein, L., Lee, Evon Batey, Akers, J., Sohl, Kristin…Beversdorf, D.Q. (2016). Psychophysiological associations with gastrointestinal symptomatology in autism spectrum disorder. *Autism Research*. Advance online publication. doi: doi:10.1002/aur.1646

time (Toichi & Kamio, 2003). These findings suggest an enhanced stress response in ASD relative to typically developing controls. Examination of heart rate variability (HRV) in the time domain, or the variation between heart beats over time, yields information on the modulation of sympathetic and parasympathetic inputs to the sinoatrial node of the heart. Studies have shown low cardiac vagal tone at rest in individuals with ASD relative to controls (Ming, JuLu, Brimacombe, Conner, & Daniels, 2005; Toichi & Kamio, 2003), suggesting altered parasympathetic tone. Taken together, these psychophysiological studies suggest a hyporesponsive parasympathetic system in ASD, with some associated changes in the sympathetic system as well (Kushki et al., 2013; Neuhaus, Bernier, & Beauchaine, 2014).

In the general population, there is a strong relationship between psychological and physical stress and GI disorders, and this may interact directly with gut bacteria to increase bacterial growth and infectivity (Lyte, Vulchanova, & Brown, 2011). Stress activates the hypothalamic-pituitary-adrenal axis, resulting in the neuronal release of catecholamines, activating the sympathetic nervous system (Elenkov & Chrousos, 2006), which has been shown to affect the gut mucosa (Lyte, et al., 2003). Sympathetic efferents can inhibit gut motility (Lomax, Harkey, & Furness, 2010; Hirst & McKirdy, 1974), suggesting a mechanism for constipation. This may involve bidirectional communication between the enteric nervous system, the intrinsic, reflexive nervous system of the GI tract, and the central nervous system. Increased sympathetic functioning and decreased parasympathetic functioning have both been noted in individuals with constipation predominant irritable bowel syndrome (IBS) (Mazur Fugala, Jablonski, Mach, & Thor, 2012), in association with a range of autonomic disturbances in IBS (Martinez-Martinez,

Mora, Vargas, Fuentes-Iniestra, & Martinez-Lavin, 2014; Pellissier, Dantzer, Canini, Mathieu, & Bonaz, 2010), though this literature is still evolving (Mazurak, Sere- dyuk, Sauer, Teufel, & Enck, 2012). Diarrhea can also be frequently observed, however, as part of the stress reaction, which includes sympathetic activation, in patients with irritable bowel syndrome (Bouchoucha, Hejnar, Devroede, Babba, & Benamouzig, 2013). Despite the literature describing alterations in both ANS and GI function in ASD, little is known about the relationship between these two systems in ASD.

Altered autonomic functioning in ASD may play a role in the etiology of GI disorders in ASD. GI disturbance, however, may also impact ANS function. In the present study, our aim was to investigate the relationship between GI symptoms and psychophysiological measures of autonomic functioning at rest and during challenge by mild stressors in children and adolescents with a confirmed diagnosis of ASD. To our knowledge, the relationship between GI symptoms and markers of autonomic function has not been studied previously in this population. Given the presence of autonomic disturbances in ASD, and the prevalence of GI disorders in ASD, understanding this relationship in the ASD population is an important exploratory first step in identifying potentially salient biomarkers that may impact treatment approaches. To explore this relationship, we examined sympathetic and parasympathetic correlates of GI symptoms by measuring HRV and skin conductance level (SCL) in a large sample of children and adolescents with ASD with varying degrees of GI dysfunction. To gain a better understanding of the impact of this association, we also explored the relationships among GI symptoms, adaptive functioning, and other co-occurring symptoms. Given the high frequency of constipation in ASD (McElhanon et al., 2014, Buie et al., 2010),

parasympathetic alterations in ASD (Kushki et al., 2013; Neuhaus et al., 2014), and the finding of decreased parasympathetic functioning in those with IBS without ASD, we predicted that decreased parasympathetic activity, both at baseline and in response to mild stress, will be associated with greater lower GI tract symptoms, especially constipation. Furthermore, due to the relationship between stress and upper GI tract problems in the general population such as gastroesophageal reflux disease (Perlman et al., 2011) and Crohn's disease (Stasi & Orlandelli, 2008), we hypothesized that positive relationships would exist, both at baseline and in response to mild stress, between sympathetic markers of stress and upper GI tract symptoms.

Methods

Participants. Children and adolescents were recruited through the Autism Speaks Autism Treatment Network (AS-ATN) registries at the University of Missouri Thompson Center for Autism and Neurodevelopmental Disorders in Columbia, Missouri and the Vanderbilt Kennedy Center and Monroe Carrell Jr. Children's Hospital at Vanderbilt University in Nashville, Tennessee. To expand the sample, additional individuals were recruited outside of the AS-ATN registry at both sites. Individuals were included in the study if they were between the ages of 6 and 18 years and had a diagnosis of ASD. All participants were diagnosed with ASD based on the *Diagnostic and Statistical Manual for Mental Disorders IV-TR* criteria (American Psychiatric Association, 2000) and administration of the Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 1989) to verify diagnosis. Individuals were excluded from the study if they had a known metabolic, genetic, or bleeding disorder.

Individuals that provided previous consent to be contacted about participating in

research studies and that met inclusion and exclusion criteria were initially recruited by telephone or e-mail. Those interested in participating were administered the complete Questionnaire on Pediatric Gastrointestinal Symptoms – Rome III (QPGS) (Walker, Caplan-Dover, & Rasquin-Weber, 2006). The phone screen QPGS used parent-report to assess the frequency, severity, and duration of GI symptoms on a 5-point scale, in addition to several Yes/No questions regarding the presence or absence of specific symptoms. An effort was made to recruit an equal number of participants with and without a GI disorder at each study site. A total of 80 participants were recruited at the University of Missouri, and 40 at Vanderbilt University, for an overall total of 120 participants. A summary of the participant demographics is shown in Table 3.1.

		% (*n*) Mean (std)	Range	*N*
Male gender		90.0% (108)		120
Caucasian, including multiracial		92.5% (111)		120
Age at consent (years)		11.8 (3.8)	6–18	120
FSIQ		84.0 (22.6)	36–130	100
Vineland Standard Score	Composite	72.3 (12.0)	45–111	83
	Communication	74.4 (14.6)	44–129	83
	Daily Living Skills	77.4 (14.3)	33–114	83
	Socialization	71.2 (13.3)	40–103	83
ABC (calculated)	Irritability	12.6 (10.4)	0–42	117
	Lethargy	10.2 (8.7)	0–43	117
	Stereotypy	5.3 (4.9)	0–21	117
	Hyperactivity	16.7 (11.9)	0–46	117
	Inappropriate speech	3.8 (3.1)	0–11	117
CSHQ total score		45.5 (8.9)	31–71	86
Upper GI tract score		4.9 (5.4)	0–24	120
Lower GI tract score		17.9 (12.4)	1–48	120
Rome III diagnoses	Functional constipation	42.5% (51)		120
	Irritable bowel syndrome	11.7% (14)		120
	Lower abdominal pain associated with bowel symptoms	9.2% (11)		119
	Upper abdominal pain associated with bowel symptoms	7.5% (9)		120
	Aerophagia	5.8% (7)		120
	Abdominal migraine	5.0% (6)		119
	Functional abdominal pain	3.3% (4)		120
	Nonretentive fecal incontinence	3.4% (4)		119

(FSIQ: Full Scale Intelligence Quotient; ABC: Aberrant Behavior Checklist; CSHQ: The Children's Sleep Habits Questionnaire.

Table 3.1. Demographic characteristics of the sample.

Assessment of gastrointestinal symptomatology. To allow for the analysis of GI symptoms on a continuum, the QPGS was scored for each participant using a scoring rubric created by the research team. The multiple-choice responses to the questions pertaining to the ten functional pediatric GI disorders assessed by the QPGS

were assigned ratings, and a quantitative score was created by summing over the ratings (scored on scales of 1–3, 0–4, 1–5, or 0–5, in accordance with the QPGS scoring criteria for each designated item; Yes/No responses were assigned 1 point each). Separate scores were summed for upper and lower GI tract disorders to study their psychophysiological profiles independently (see Table 3.2 for a list of the QPGS upper and lower GI symptoms assessed in this study). Furthermore, items that were included multiple times through- out the scoring rubric for different GI disorders (e.g., item A1, "upper abdominal pain or discomfort 'several times a week' or more often," is scored 4 times through- out the questionnaire, each contributing to different categories of functional pediatric GI disorders, such as Functional Abdominal Pain and Irritable Bowel Syndrome) were only scored once. For items where lower numbers indicated greater severity, the scoring was reversed such that greater scores indicated greater severity (i.e., items A6, B5, C1, C2). Items answered as "It depends" or "Don't know" were scored as missing. These quantitative scores represented the duration, frequency, and severity of upper and lower GI tract symptomatology (See Table 3.2 for the complete scoring rubric). Given the age range of the participants and varying levels of verbal and cognitive functioning, the parent-report forms were administered to most families, and were completed by the participant's caretaker. In four higher functioning individuals 17 years

of age and older, where the parent indicated the participant would give the most reliable response, the child/adolescent self-report form was completed by the participant. The QPGS has been shown to be a reliable measure of functional GI disorders (Van Tilburg, Squires, Blois-Martin, Leiby, & Langseder, 2013), and Rome III criteria show adequate construct validity (Saps et al., 2014).

Psychophysiology protocol. To examine ANS functioning and reactivity to stress, heart rate variability (pNN50, as described below, to assess parasympathetic modulation of cardiac activity) (Kleiger, Stein, & Bigger, 2005; Task Force, 1996) and skin conductance level, (as described below, to assess sympathetic nervous system activity) (Lidberg & Wallin, 1981) were collected. A BIOPAC MP150 modular data acquisition and analysis system attached to a laptop computer was used to collect all psychophysiology data (BIOPAC Systems, Inc., Goleta, CA). Electrocardiogram (ECG) data were collected utilizing a BIOPAC ECG-100C amplifier outfitted with an MEC110C module extension cable and LEAD110 electrode leads (BIOPAC Systems, Inc., Goleta, CA) attached to a MP150 data acquisition system. The ECG-100C amplifier was set at a gain of 1000, and a low pass filter of 0.05 Hz. Participants were outfitted with a 2-lead ECG setup consisting of BIOPAC EL503 Ag/AgCl disposable electrodes with a moderate adhesive backing for contact with the skin. One lead was placed below the right clavicle, in the mid-clavicular line within the frame of the rib cage, and the other on the lower left abdomen within the rib cage frame. A ground was obtained through the VIn- connection on the EDA100C amplifier. After placing electrodes on the participant, the ECG signal was verified by observing a series of characteristic QRS complexes. Electrodes were replaced on the participant if the initial ECG signal was not suitable for

analysis. Skin conductance level (SCL) data were collected using two reusable skin conductance transducers filled with isotonic gel connected to a BIOPAC GSR-100 amplifier attached to the MP150. Transducers were placed on the distal phalanges of the participant's hand to measure SCL. All psychophysiology data were acquired using AcqKnowledge Data Acquisition and Analysis Software Version 4.2 (BIOPAC Systems, Inc., Goleta, CA).

Stress reactivity protocol. Participants were seated at a table, directly opposite the investigator. Participants who were not able to remain seated at the table by themselves were permitted to sit in their caregiver's lap for the duration of the study or until they felt comfortable being seated on their own. Seven participants were excluded from the study for not being able to comply with these instructions. At the beginning of the stress reactivity protocol, the researcher instructed the participant to sit still, remain quiet, and breathe normally during which 3 min of baseline ECG and SCL data were collected. Next, the participant engaged in either vibrotactile or cold pressor stimulation to the hands in a counter-balanced fashion, where the order of vibrotactile and cold pressor stimulation was reversed after every 10 participants to account for potential order effects. Although the cold pressor test has been established in the research literature as a method for eliciting a momentary increase in sympathetic nervous activity, (Zvan, Zaletel, Pretnar, Pogacnik, & Kiauta, 1998), we wished to also test the effects of vibrotactile stimulation to the hands given a recent report suggesting that vibrotactile stimulation can elicit changes in heart rate and blood pressure, indicating an increase in sympathetic nervous system activity (Foster et al., 2013). This condition was also implemented as a secondary stressor given the expectation that some children would not

tolerate the cold pressor test. For the vibrotactile condition, participants were instructed to grasp a vibrating stimulator (Conair WM200X, Stamford, CT) that was held by the researcher at an approximate height of the participant's chest by placing the palmar surface of their hand on the middle of the stimulator and wrapping their fingers around the top edge of the device. The stimulator was then placed on "high," and ECG data were collected for 30 sec. The stimulator produces 80Hz oscillations at 1-mm amplitude on the "high" setting. Since the vibrotactile stimulator would result in artifact during SCL data collection, SCL data were not analyzed for the vibrotactile stimulation condition. However, the skin conductance transducers remained on the participant's fingers as this connection provided the electrical ground for the ECG amplifier. Immediately following this procedure, the stimulator was removed from the participant's hand, and a 3-min rest period was initiated. After the 3-min rest period, the skin conductance transducers were moved to the opposite hand, and the vibrotactile protocol explained above was repeated, with a 3-min rest period after vibrotactile stimulation. For the cold pressor test, a cooler was calibrated to a target temperature of 4°C using ice and tap water. To encourage test compliance, a small yellow rubber duck was placed in the cooler, and participants were instructed to press the duck to the bottom of the cooler with their hand and hold it down for 30 sec. ECG and SCL data were collected from the opposite hand for 30 sec., immediately followed by a 3-min rest period. After the rest period, the skin conductance transducers were switched to the participant's other hand, and the cold pressor test was repeated as above. A timeline of the order of tasks in the stress reactivity protocol is demonstrated in Figure 3.1 for the vibrotactile stimulation-first condition.

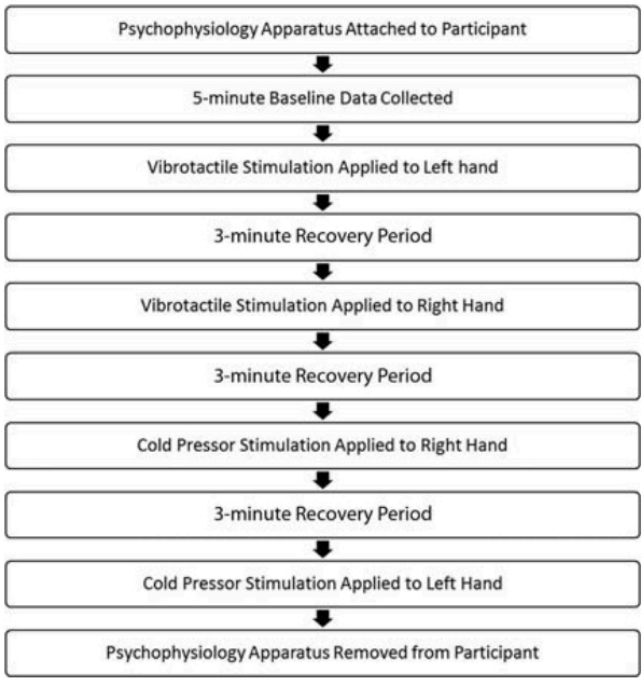

Figure 3.1. Illustration of the order of tasks in the stress reactivity protocol, for the vibrotactile stimulation-first condition.

Additional measures. The participant's caretaker completed the Vineland Adaptive Behavior Scales, Second Edition (Vineland-II) (Sparrow, Cicchetti, & Balla, 2005), Aberrant Behavior Checklist (ABC) (Aman, Singh, Stewart, & Fields, 1985), the Sensory Over-Responsivity Scale (SensOR) (Schoen, Miller, & Green, 2008), the Children's Sleep Habits Questionnaire (CSHQ) (Owens, Spirito, & McGuinn, 2000) and provided a self-reported/caregiver-reported list of co-occurring disorders from the Autism Treatment Network Parent Baseline Questionnaire (Table 3.3). During the study visit, participants also provided blood and saliva samples after the initial psychophysiology baseline recording and provided a second saliva sample at the conclusion of the study, for use in a separate study. Participants were given approximately 10 min to rest and consume a small snack after the blood draw.

Data processing and statistical methods. ECG data were visually inspected, and records with excessive motion artifacts were excluded from further analysis. Data were then imported into Kubios HRV, Version 2.2 (Tarvainen, Niskanen, Lipponen, Ranta-aho, & Karjalainen, 2014). R-R intervals, or the amount of time between heart beats, were determined by QRS detection, and were then visually inspected for errors. The R-R interval is influenced by vagal nerve (i.e., parasympathetic) activity (Katona, Poitras, Barnett, & Terry, 1970), and increases in the R-R interval are associated with increases in parasympathetic tone, whereas decreases in the R-R interval are associated with decreases in parasympathetic tone. Increases and decreases in parasympathetic activity to the heart create beat-to-beat variations in heart rate, termed heart rate variability (HRV). For the present study, HRV was assessed by determining the percentage of pairs of consecutive R-R intervals that differed by more than 50ms, widely known as pNN50 (Bigger et al., 1988). pNN50 values were calculated for the initial baseline reading and each vibrotactile and cold pressor stimulus condition. Mean SCL data in microsiemens (μS) were determined for each baseline and the cold pressor condition using AcqKnowledge software, Version 4.2 (BIOPAC, Goleta, CA). The data were visually inspected by a study team member with extensive experience in ECG and SCL data collection and analysis, and those with excessive artifacts due to motion were removed from further analysis.

Statistics. The upper and lower GI tract sum scores, as previously described, represent GI symptomatology. To normalize the distribution of the psychophysiological variables, the pNN50 data were root-arcsine transformed and the mean RR data were log transformed. Three variables were analyzed for each psychophysiological endpoint: first

baseline, a cold pressor minus baseline change score, and a vibrotactile minus base- line change score. As previously mentioned, the vibrotactile condition was not available for the skin conductance endpoint due to artifact from the vibrotactile stimulator.

The relationships between GI scores and psychophysiological variables, as well as secondary behavioral variables such as ABC and Vineland, were assessed through Pearson partial correlations controlling for age and gender. ANOVA was used to assess differences in GI scores when key comorbidities were reported, and Cohen's *d* is reported as a measure of effect size. Behavioral variables and key comorbidities were considered candidate effect modifiers of the associations between GI scores and psychophysiological variables; these relationships were tested using likelihood ratio tests on interaction terms in multiple linear regression.

Nonparametric versions of some of the statistical tests described above and modifications to the GI scoring algorithm were performed to assess the sensitivity of our results to model assumptions and missing data in the QPGS. Modifications to the GI scoring algorithm utilized to account for the effect of missing data included (a) defining the score as the mean of the non-missing items instead of the sum, (b) excluding participants with more than three missing items, (c) a square-root transform of the sum-score, and (d) removal of several large outliers in the mean R-R data. Conclusions did not differ, and results are not reported. Finally, a significance threshold of 0.05 was used to report findings, and the issue of reporting and interpreting P-values considering the multiple comparisons problem is discussed further in the Discussion section.

Results

Participants. Of the 120 participants, 108 (90%) were male, the average age was 11.8 years (*SD*=3.8), and the average full scale intelligence quotient (FSIQ) from their AS-ATN data (performed by trained psychometricians at each ATN site, specific IQ tests chosen based on the participant's verbal ability) was 84.0 (*SD* = 22.6), as observed in Table 3.1, where average scores on adaptive behavior and aberrant behavior scales are also noted. The most frequent gastrointestinal disorders present in the sample were functional constipation (42.5%), lower abdominal pain associated with irritable bowel symptoms (9.2%) and upper abdominal pain associated with irritable bowel symptoms (7.5%) per Rome III criteria. Primary analyses therefore focused on lower GI tract symptoms.

A total of 10 participants were excluded from all ANS analyses due to motion artifact or lack of protocol compliance. For the vibrotactile and cold pressor stimulus conditions, a total of 109 and 106 participants were included in the ECG analyses, respectively. After exclusion for motion artifact on the cold pressor stimulus condition, analysis for SCL included a total of 84 participants.

Psychophysiological markers and lower GI tract symptomatology. For lower GI tract score, a significant positive relationship between QPGS lower GI tract score and the primary parasympathetic marker, baseline pNN50, was observed while controlling for age and gender, $p = 50.039$, $r = 50.20$, 95% CI [0.01, 0.37]. A significant negative relationship between QPGS lower GI tract score and the pNN50 change score for the cold pressor condition was observed, $p = 0.015$, $r = 20.24$, 95% CI [20.41, 20.05]. Thus, subjects with worse lower GI symptoms (a higher score for lower GI) tend to have

greater parasympathetic tone at baseline, but lower parasympathetic tone change-score in response to cold pressor stimulation. There was no significant relationship with the change score for the vibrotactile condition. The correlations between lower GI tract symptomatology and the mean RR interval and SCL variables were low in magnitude and not statistically significant (Table 3.3).

	Lower GI tract score			Upper GI tract score		
Endpoint	Correlation (95% CI)	*P*-value	*N*	Correlation (95% CI)	*P*-value	N
pNN50: First baseline	**0.20 (0.01, 0.37)**	**0.0394**	110	0.11 (−0.08, 0.29)	0.2712	110
pNN50: Vibrotactile-baseline	−0.13 (−0.31, 0.07)	0.1932	109	0.08 (−0.11, 0.26)	0.4276	109
pNN50: Cold Pressor-baseline	**−0.24 (−0.41, −0.05)**	**0.0150**	106	−0.11 (−0.30, 0.08)	0.2526	106
meanRR: First baseline	−0.01 (−0.20, 0.18)	0.9292	110	−0.03 (−0.22, 0.16)	0.7534	110
meanRR: Vibrotactile-baseline	0.04 (−0.15, 0.23)	0.6966	109	0.16 (−0.03, 0.34)	0.0968	109
meanRR: Cold pressor-baseline	−0.11 (−0.30, 0.08)	0.2597	106	−0.01 (−0.20, 0.19)	0.9407	106
Skin conductance: First baseline	0.15 (−0.07, 0.35)	0.1829	84	0.12 (−0.10, 0.33)	0.2798	84
Skin conductance: Cold pressor-Baseline	−0.14 (−0.35, 0.09)	0.2278	78	−0.17 (−0.38, 0.06)	0.1391	78
Vineland socialization score	−0.09 (−0.30, 0.13)	0.4354	83	−0.07 (−0.29, 0.15)	0.5163	83
Vineland daily living skills score	−0.08 (−0.29, 0.14)	0.4828	83	−0.08 (−0.29, 0.14)	0.4661	83
Vineland composite score	−0.12 (−0.33, 0.10)	0.2979	83	−0.12 (−0.33, 0.10)	0.2959	83
Vineland communication score	−0.10 (−0.31, 0.12)	0.3922	83	−0.16 (−0.37, 0.06)	0.1500	83
FSIQ	−0.00 (−0.20, 0.20)	0.9870	100	−0.10 (−0.30, 0.10)	0.3070	100
CSHQ total score	**0.37 (0.17, 0.54)**	**0.0005**	86	**0.33 (0.12, 0.51)**	**0.0020**	86
ABC stereotypy	0.08 (−0.10, 0.26)	0.3919	117	0.02 (−0.17, 0.20)	0.8582	117
ABC lethargy	−0.02 (−0.20, 0.16)	0.8221	117	0.15 (−0.04, 0.32)	0.1163	117
ABC irritability	**0.20 (0.01, 0.37)**	**0.0346**	117	*0.16 (−0.03, 0.33)*	*0.0928*	117
ABC inappropriate speech	0.00 (−0.18, 0.19)	0.9694	117	0.11 (−0.08, 0.28)	0.2535	117
ABC hyperactivity	0.13 (−0.05, 0.31)	0.1525	117	0.14 (−0.04, 0.31)	0.1360	117

[Partial Correlations Controlled for Age and Gender pNN50 and meanRR Variables Transformed Prior to Analysis. Vineland Scores are Standard Scores and ABC Scores are Calculated].

pNN50: percentage of normal R-R intervals that differed by 50 milliseconds or more; meanRR: average amount of time between R-R intervals; FSIQ: full-scale intelligence quotient; CSHQ: The Children's Sleep Habits Questionnaire; ABC: Aberrant Behavior Checklist.

Bold type for $P < 0.05$, italicized for $P < 0.1$.

Table 3.3. Pearson Partial Correlations Between lower and upper GI tract Scores and Endpoints

An individual's degree of reactivity to either the vibrotactile or the cold pressor condition may be highly dependent upon sympathetic and parasympathetic baseline tone. If a participant enters the testing environment with in a heightened state of arousal, then reactivity to these stimuli may be reduced. To assess the potential for this possible dependent relationship, we examined the correlation between baseline pNN50 and pNN50 change score for the cold pressor condition. A significant inverse correlation was

observed, $p < 0.001$, $r = 20.49$, 95% CI [20.63, 0.33], consistent with the possibility that reactivity to cold pressor might be dependent on baseline parasympathetic tone.

It is possible that participants taking medications that interact with gut motility, the adrenergic system, or the serotonergic system (i.e., stimulants, alpha-2 agonists, beta-adrenergic antagonists, neuroleptics, antidepressants, antiepileptics, or drugs directly impacting gut motility) could be a potential confound. Thus, participants taking the aforementioned medications were removed, and separate analyses were conducted. After removal of these participants, the relationship between lower GI tract score and pNN50 change score for the cold pressor condition remained $r = 20.35$, 95% CI [20.59, 20.05] with n = 44), and a trend emerged for a relationship with the pNN50 change score for the vibrotactile condition ($p = 0.062$, $r = 20.28$, 95% CI [20.54, 0.02] with n = 45). The relationship with baseline pNN50 was no longer statistically significant ($p = 0.131$, $r = 0.23$, 95% CI [20.07, 0.50] with n = 45) in the reduced sample, however the magnitude of the correlation remained the same.

Psychophysiological markers and upper GI tract symptomatology. For upper GI tract symptomatology, no significant relationships were detected with any of the psychophysiological variables. Relationships with baseline pNN50 and the pNN50 cold pressor change score were not significant. Thus, the stress response at baseline and in response to stressors is not related to upper GI tract symptoms in this sample.

GI symptomatology and sleep, adaptive behaviors, and aberrant behaviors. Significant positive relationships were observed between lower GI tract symptomatology and the ABC irritability subscale ($p = 0.035$, $r = 0.20$, 95% CI [0.01, 0.37]) and CSHQ total score ($p < 0.001$, $r = 0.37$, 95% CI [0.17, 0.54]). These relationships were also

observed with the upper GI tract symptomatology (a trend for ABC Irritability: $p = 0.093$, $r = 0.16$, 95% CI 20.03, 0.33], and significant for CSHQ total score: $p = 0.002$, $r = 0.33$, 95% CI [0.12, 0.51]). ABC hyperactivity significantly modified the relationship between lower GI tract symptomatology and the pNN50 cold pressor change score ($p = 0.028$ for the interaction term). The negative association between lower GI tract symptomatology and the cold pressor change score is greatest among those with low hyperactivity scores and attenuates as hyperactivity increases, suggesting that hyperactive participants may not be as sensitive to changes in parasympathetic stimulation. A summary of the findings can be found in Table 3.3. Sleep, adaptive behavior, and other aberrant behavior measures were not found to modify any of the significant relationships between GI symptomatology and the psychophysical markers described above.

GI symptoms and co-occurring medical or psychiatric symptoms. Co-occurring diagnoses were documented in the AS-ATN record for most participants. Our interest focused on anxiety symptoms, while also examining ADHD, depression, regression/loss of skills and seizures. Individuals with anxiety disorder (Cohen's $d = 0.66$), depression (Cohen's $d = 0.45$), loss of skills/regression (Cohen's d=0.95), or seizures (Cohen's $d = 0.83$) had significantly higher upper GI tract scores, and individuals with loss of skills/regression (Cohen's $d = 0.59$) had significantly higher lower GI tract scores (Table 3.4).

Comorbidity	Frequency	Mean lower GI tract score			Mean upper GI tract score		
		Present	Absent	*P*-value	Present	Absent	*P*-value
Attention deficit hyperactivity disorder	42.5%	*20.4*	*16.2*	*0.067*	5.8	4.3	0.140
Anxiety disorder	35.8%	19.7	17.0	0.261	**7.1**	**3.7**	**<0.001**
Depression	28.3%	19.2	17.5	0.495	**6.6**	**4.2**	**0.027**
Loss of skills/regression	30.8%	**22.8**	**15.8**	**0.003**	**8.2**	**3.4**	**<0.001**
Seizures	13.3%	21.2	17.4	0.263	**8.7**	**4.3**	**0.003**

Presence/Absence Classified as Yes vs. No/Unknown ANOVA *P*-values.
Bold type for $P < 0.05$, italicized for $P < 0.1$.

Table 3.4. Comorbidity Status and Mean GI Scores by Presence/Absence of Comorbidity

Furthermore, presence or absence of anxiety symptoms was found to be a significant effect modifier for the relationship between lower GI tract score and pNN50 Baseline ($p = 0.035$ for the interaction term). This interaction is illustrated in Figure 3.2A: the slope of the regression line for lower GI tract score on baseline pNN50 is near zero among individuals without anxiety disorder (slope = 3.80) and relatively steep among those with anxiety disorder (slope = 27.60), suggesting that individuals with anxiety symptoms are at an increased risk for lower GI tract symptoms, particularly in the setting of greater parasympathetic tone. The presence or absence of a history of regression/loss of skills was also found to modify the relationship between lower GI tract score and base- line pNN50 ($p= 0.016$ for the interaction term, slope in the absence of regression/loss of skills=2.73, slope in presence of regression/loss of skills = 30.49, see Figure 3.2B), suggesting that individuals with regressive autism are at an increased risk for lower GI tract symptoms, particularly in the setting of greater parasympathetic tone.

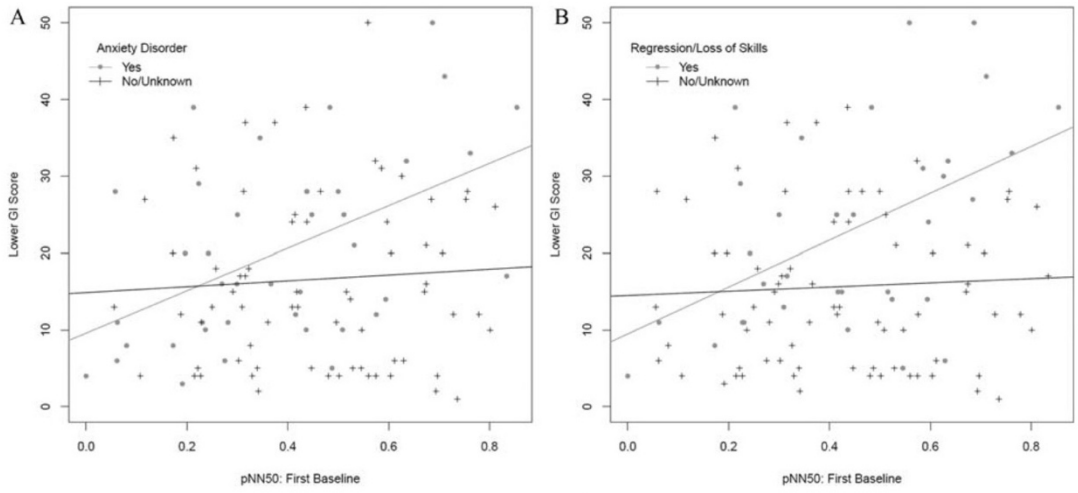

Figure 3.2. Impact of effect modifiers on the ANS-gastrointestinal symptomatology relationships. **(A)** Effect of presence or absence of anxiety on the relationship between lower GI tract scores and pNN50 baseline. **(B)** Effect of presence or absence of history of regression/loss of skills on the relationship between lower GI tract scores and pNN50 baseline.

Discussion

This is the first study to explore the relationship between gastrointestinal symptoms and ANS functioning in individuals with ASD. We observed a significant correlation between lower GI tract symptoms and both the pNN50 from the ECG data at rest and the change in pNN50 with cold pressor stimulation. These results were interrelated, however, with the cold pressor findings possibly driven by the variation in baseline pNN50. However, with strict exclusion of potential confounding medications, the cold pressor change in pNN50 relation- ship remained significant while the baseline pNN50 was no longer significant with this smaller sample, suggesting that reactivity to stress may be an independent factor of relevance in this effect. As the pNN50 is a marker of parasympathetic function, this supports the hypothesis that lower GI tract symptoms are related to parasympathetic activity in the ASD population. A significant amount of the lower GI tract symptoms reported in the population studied herein were constipation

symptoms, consistent with previous work (Gorrindo et al., 2012; McElhanon et al., 2014), suggesting a relationship between constipation and parasympathetic tone in ASD. Furthermore, this finding agrees with previous research suggesting impaired parasympathetic functioning and sympathovagal balance in those with irritable bowel syndrome without ASD, although for constipation-predominant irritable bowel syndrome, vagal dysfunction had been most prominent (Liu, Wang, Yan, & Chen, 2013). Future studies should explore whether the relationship between autonomic functioning and lower GI tract symptoms differs in those with ASD as compared to those without ASD, to determine whether this relationship is generalized to all participants with lower GI tract symptoms.

Given the correlational nature of this study, it is not possible to assess the causality of this association. It is tempting to believe that parasympathetic tone affects lower GI tract symptoms, as has been suggested in gastrointestinal disorders in those without ASD (Pellissier et al., 2010), but it is also possible that feedback from a constipated GI tract could affect parasympathetic tone in ASD. One opportunity to evaluate a possible causal relationship between parasympathetic tone and lower GI tract symptoms would be exploring the relationship between parasympathetic tone and response to standard treatment for constipation. If successful treatment of constipation leads to diminished parasympathetic tone, feedback from the gut is likely affecting this element of the ANS. By contrast, parasympathetic tone could predict who will and will not respond to standard constipation treatment, which might serve as an important biomarker and guide treatment selection.

For upper GI tract symptomatology, no significant relationships were observed

with the psychophysiological variables; although one contributing factor could be the small number of participants with upper GI tract disorders (19.2%). Future studies targeting greater numbers of patients with upper GI tract problems would be needed to more conclusively address a relationship between ANS variables and upper GI tract symptomatology.

A relationship between GI disturbances and irritability and sleep problems in ASD is not entirely surprising, as pain resulting from constipation or other abdominal distress would likely affect behavior and sleep (Buie et al., 2010). In the exploration of these relationships in our study, upper and lower GI tract symptomatology were both independently found to relate to both irritability and sleep. Other co-occurring symptoms have been previously associated with GI disorders in ASD (Peters et al., 2014; Mazurek et al., 2013). Physiological hyperarousal has been shown in anxiety disorders, such as elevated heart rate and reduced respiratory sinus arrhythmia (a measure of parasympathetic tone) at baseline (Thayer, Friedman, & Borkovec, 1996), and failure to reduce sympathetic tone as evidenced by reduced declines skin conductance level during the day- time and in bed at night (Roth et al., 2008). Other studies found similar cardiac findings in those under stress, suggesting decreased parasympathetic control (Brosschot, Gerin, & Thayer, 2006). Furthermore, anxiety disorders commonly co-occur in ASD (van Steensel, Bögels, & Perrin, 2011), and studies have shown similar physiological alterations such as elevated basal heart rate (Kushki et al., 2013), and autonomic hyperarousal at baseline and in response to social anxiety and social cognition (Kushki, Brian, Dupuis, & Anagnostou, 2014). In the present study, an association with anxiety was identified and found to represent a significant modifier of the association between

pNN50 and lower GI tract scores. This suggests that individuals with ASD and anxiety disorders may be at an increased risk of lower GI problems, and that the mechanism by which this occurs is an enhanced stress response. Previous research has found anxiety to be associated with a range of gastrointestinal problems in ASD including constipation (Mazurek et al., 2013). In our study, specifically, base- line pNN50 was strongly related to lower GI tract scores only in participants whose caretakers reported the presence of an anxiety disorder (Figure 3.2A).

In addition, greater upper GI tract scores were associated with history of regression, and seizures, while lower GI tract scores were also associated with regression history. An association between gastrointestinal disturbances in general and seizures has also been previously observed in a recent electronic health record time-series analysis (Doshi-Velez et al., 2014). The observed relationship with a history of regression is of some interest. The relationship between pNN50 and lower GI tract symptomatology was significantly stronger in participants with a reported history of regression or loss of skills (Figure 3.2B). The etiology of regression and loss of skills in ASD remains very poorly understood. One previous study had reported abnormal stool patterns in individuals with ASD with regression (Valicenti-McDermott et al., 2008). These results suggest further exploration of the autonomic and gastrointestinal systems is needed in children with a history of regression.

There are several important limitations in this study. First, few participants had upper GI tract diagnoses, and many participants scored a “0” for upper GI tract symptomatology on our quantitative measure derived from the QPGS, limiting the conclusions that can be drawn regarding upper GI tract symptoms. A future study may

need to specifically recruit participants with upper GI tract symptomatology to address this challenge. Second, the use of the QPGS as a continuous measure allowed us to evaluate correlations with ANS biomarkers and co-occurring symptoms, but this differs from its designed use to determine if an individual meets criteria for functional GI disorders. Another potential limitation is the fact that GI symptoms were assessed by self- or parent-report on the QPGS. This may be problematic for individuals with ASD who have limited expressive language and whose parents may not be aware of their child's GI pain or potential discomfort. However, previous research has demonstrated a direct correlation between parent report and true GI symptoms (Gorrindo et al., 2012). Alternative approaches to supplement this type of information with direct assessment by a gastroenterologist should be considered in future work. Regardless, use of the QPGS in this study is a strength given that the measure is standardized and reliable (Van Tilburg et al., 2013) and Rome III criteria display construct validity (Saps et al., 2014). It is also possible that dietary restrictions, food preferences, or utilization of complementary alternative treatments, which were not assessed, could also have impacted the results. Finally, the sample size is modest and we chose to present uncorrected *p*-values given the exploratory nature of the research. The significant findings in this study may be most useful as hypotheses for future studies, including studies exploring whether these findings serve as markers that predict response to standard treatment, and for studies examining the co-occurring disorders as they may also have important implications for treatment of specific ASD subgroups.

A final but important limitation in the present study is that the enteric nervous system was not directly studied. The activity of the gut is modulated by both

preganglionic sympathetic and parasympathetic neurons. Generally speaking, stimulation of sympathetic neurons inhibits gut motility, whereas stimulation of parasympathetic neurons allows digestive activities, there are extensive, bidirectional connections between the CNS and the ENS, providing multiple potential pathways for an interaction between stress, the brain, and the gut. As such, future research should examine ENS and CNS-ENS interactions and their influences on constipation in ASD.

Despite these limitations, the primary finding of a relationship between parasympathetic psychophysical markers and lower GI tract symptoms, moderated by anxiety in the ASD population, may lead to better understanding of why constipation is so problematic in these individuals. Follow-up studies could establish the directionality of this association by assessing whether treatment of constipation results in a change in parasympathetic tone. Likewise, GI symptoms could be re-evaluated after successful treatment of anxiety in the ASD population. Future work will be necessary to see how these factors relate to regression and loss of skills. Targeted work in individuals with ASD and upper GI tract symptoms will be necessary to address these questions more robustly; although the low rate of these problems in our population may suggest that they are not enriched in the ASD population. Subsequent work could also explore how these findings in the ASD population compare to findings in individuals without ASD.

CHAPTER FOUR:

ASSOCIATIONS BETWEEN CYTOKINES, ENDOCRINE STRESS RESPONSE, AND GASTROINTESTINAL SYMPTOMS IN ASD[1]

Introduction

Many studies suggest an increased prevalence of GI problems in individuals with ASD relative to typically-developing individuals (McElhanon et al., 2014; Chaidez et al., 2014), especially for constipation, but the cause of this relationship is not currently known. Both acute and chronic stress are associated with the onset of GI disease as well as exacerbation of GI symptoms (Dinan et al., 2006). Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, which results in a cascade of neuroendocrine factors that modulate stress reactivity, digestion, and immunological functioning. Activation of the HPA axis in individuals with irritable bowel syndrome (IBS) without ASD is associated with an augmented adrenocorticotropic hormone (ACTH) and cortisol response, as well as increased sympathetic activity and low vagal tone (Aggarwal et al., 1994; Mayer, 2000). Furthermore, a generalized increase in the stress response is also characteristic of those with ASD relative to typically developing individuals (Ming et al., 2005; Mazurek et al., 2013; Spratt et al., 2012). However, the relationship between the stress response and GI symptoms in ASD is poorly understood.

In addition to core ASD symptoms, many children with ASD have associated co-occurring symptoms, including anxiety, agitation, irritability, and aggression. Among individuals with ASD, GI symptoms predict increased behavioral symptoms in some

[1] Ferguson, B.J., Marler, S., Altstein, L.L., Batey, E.B., Mazurek, M.O., McLaughlin, A., …Beversdorf, D.Q. (2016). Associations between cytokines, endocrine stress response, and gastrointestinal symptoms in autism spectrum disorder. *Brain, Behavior, and Immunity*. Advance online publication. doi: 10.1016/j.bbi.2016.009

domains, including heightened stress and anxiety, increased rigid-compulsive behavior, and irritability/agitation (Coury et al., 2012; Bishop-Fitzpatrick et al., 2015; Nikolov et al., 2009; Peters et al., 2014). Exposure to a social situation is associated with an enhanced cortisol response in ASD relative to typically- developing peers (Corbett et al., 2010), and there is a positive relationship between cortisol and self-reported social stress and anxiety during social situations in ASD (Lopata et al., 2008). Furthermore, a heightened cortisol response is linked to decreased intelligence as well as receptive and expressive language (Kidd et al., 2012), and some have proposed that the effects of stress on the HPA axis may contribute to these outcomes (Maldonado et al., 2008). In the mouse brain, elevated interleukin-6 (IL-6) levels are associated with increased autism-like features such as impairments in cognition, learning, anxiety, and social interaction, suggesting a potential cytokine of interest in ASD (Wei et al., 2012). Additionally, levels of IL-6 have been shown to be increased in individuals with regressive autism, (i.e. losing previously acquired skills such as language or social skills) (Ashwood et al., 2011). Determining biomarkers associated with GI symptoms in ASD has the potential to assist in the treatment of this and perhaps other medical and psychological complications in this population.

Activation of the HPA axis also leads to cascading immune system responses through the release of cytokines. Several studies have shown that individuals with ASD have an atypical immune response, including alterations in IL-12, IFN-c, IL-2, IL-6, IL-10, and TNF-α (Lyte et al., 2011; Goines and Ashwood, 2013). Levels of the proinflammatory cytokines IL-6 and tumor necrosis factor- alpha (TNF-α), which lead to activation of the HPA axis (Dunn, 2006), have been shown to be increased in those with

ASD (Ashwood et al., 2011; Emanuele et al., 2010; von Känel et al., 2005). In the general population, GI symptoms themselves have been reported to also be associated with alterations in TNF-α and IL-6, suggesting a potential interaction between stress and immune functioning in those with GI dysfunction (Lyte, Vulchanova, & Brown, 2011; von Känel et al., 2006). Therefore, these specific stress, ASD, and GI- associated cytokines were the focus for our study of the interaction with stress reactivity.

Thus, the goal of the present study was to examine the relationships between GI symptoms and cortisol response to stress, as well as the stress-responsive cytokines IL-6 and TNF-α. We hypothesized that GI symptoms would be positively correlated with change in cortisol concentrations after stress, as well as with levels of IL-6 and TNF-α. We also examined the relationship between psychophysiological markers of autonomic nervous system functioning and GI symptomatology, which are known to be interrelated with lower GI symptomatology in ASD (Ferguson et al., in press). Furthermore, relationships between these measures and intelligence, ASD-associated behaviors, and adaptive functioning were explored, to determine how these findings relate to other co-occurring conditions associated with GI symptomatology.

Methods

Participants. Participants were recruited through the Autism Speaks – Autism Treatment Network and through clinics at the University of Missouri Thompson Center for Autism and Neurodevelopmental Disorders in Columbia, Missouri, and the Vanderbilt Kennedy Center and Monroe Carrell Jr. Children's Hospital at Vanderbilt University in Nashville, Tennessee. A total of 120 individuals (mean age = 11.8, *SD* = 3.8, range = 6–18, mean Full Scale Intelligence Quotient = 84, *SD* = 22.6, Range = 36–

130, 111 Caucasian, 108 males) with a diagnosis of ASD participated in the study. ASD diagnosis was based on the Diagnostic and Statistical Manual for Mental Disorders IV-TR criteria (American Psychiatric Association, 2000), and diagnoses were verified by administration of the Autism Diagnostic Observation Schedule (ADOS; Lord et al., 1989). Individuals with a known mitochondrial disorder, genetic disorder such as Fragile X syndrome or tuberous sclerosis, or a bleeding disorder were excluded.

Potential participants' medical records were screened, and the parents of those who were deemed eligible to participate were contacted. An effort was made to recruit a similar number of individuals with and without GI disorders at each study site. A total of 107 and 105 individuals provided pre-stress blood samples that were suitable for analysis for IL-6 and TNF-α, respectively. A total of 81 pre-stress and 79 post-stress salivary cortisol samples were suitable for analysis.

Assessment of Gastrointestinal Symptoms. The QPGS (Walker et al., 2006) was used to assess GI symptomatology. As previously described, The QPGS is a 71-item parent report measure that assesses the frequency, severity, and duration of functional (i.e. no associated pathology observed in endoscopy, imaging, or blood) GI symptoms in children and adolescents. The QPGS has been used to assess GI dysfunction in ASD with clinician-parent agreement at 92.1% for presence of any QPGS disorder, and fair agreement for functional constipation (Gorrindo et al., 2012). Continuous variables were created for upper and lower GI tract symptoms as previously described in Chapter 2.

IL-6 and TNF-α Cytokines. Fasting blood samples were collected from participants, and sera were extracted and stored at 80°C until analysis. Cytokine concentrations were determined using Enzyme-linked Immunosorbent Assay kits

(ELISA) from R&D Systems (Minneapolis, MN). Intra-assay coefficient of variation (CV) for IL-6 and TNF-α was 2.6% and 4.7%, respectively. Inter-assay CV for IL- 6 and TNF-α was 4.5% and 5.8%, respectively. Samples were run in duplicate, and absorbance was measured using a Spectramax M3 plate reader (Molecular Devices, Sunnyvale, CA).

Stress Response Protocol. All participants at each site engaged in a stress response protocol involving momentary, unpleasant physical stimuli, as previously described in the Chapter 2.

Salivary Cortisol. Salivary cortisol samples were obtained from participants prior to the stress response protocol, and at the end of the study, with approximately 1-h between collection of the samples. Samples were collected using a SalivaBio Children's Swab (Salimetrics, Carlsbad, CA), and were analyzed using ELISA (R&D Systems, Minneapolis, MN). Intra- and inter-assay CVs for the kit were 4.6% and 6%, respectively. Samples were run in duplicate, and absorbance was measured using a Spectramax M3 plate reader (Molecular Devices, Sunnyvale, CA).

Assessments of intelligence, ASD-associated behaviors, and co-occurring conditions. The full-scale intelligence quotient (FSIQ) for each participant from the AS-ATN database (Wechsler Intelligence Scale for Children-IV, Wechsler Abbreviated Scale of Intelligence I & II, or Stanford-Binet V, each with a mean of 100 and standard deviation of 15) was used as an index of intelligence. The Aberrant Behavior Checklist, Community Version (ABC; Aman et al., 1985) was administered to the participants' caregivers to assess ASD-associated behavior problems. Adaptive behavior was assessed using the Vineland Adaptive Behavior Scales, Second Edition (Vineland-II; Sparrow et al., 2005). Key co-occurring medical and psychological disorders were assessed using the

Autism Speaks – Autism Treatment Network Health and Mental Health History Questionnaire, a yes or no format questionnaire to query history of any of the following: anxiety disorder, depression, regressive autism (i.e. loss of previously acquired skills such as language or social skills), or seizures.

Statistical Methods. The quantitative measures of upper and lower GI tract functioning derived from the QPGS represent GI symptomatology. Cortisol response is defined as the log-transformed ratio of post-stress to pre-stress cortisol and is the primary cortisol response variable of interest. Cortisol pre-stress, IL-6 and TNF-α were also log transformed prior to analysis. For the ECG data, three variables were analyzed: first baseline, a cold pressor minus baseline change score, and a vibrotactile minus baseline change score. Root-arcsine and log transformations were applied to the pNN50 and mean R-R variables, respectively, prior to analysis. For the skin conductance level data, first baseline and the cold pressor minus baseline change score were analyzed; no transformation was applied.

The relationships between the cytokine and cortisol response endpoints and GI functioning, as well as secondary variables such as FSIQ, ABC and Vineland, were assessed as Pearson partial correlations controlling for age and gender, and, in the analysis of cortisol response, pre-stress cortisol. Key co-occurring conditions mentioned above were considered candidate effect modifiers of these associations; these relationships were tested using likelihood ratio tests on interaction terms in multiple linear regression. The cytokine and cortisol endpoints were also considered candidate effect modifiers of previously reported associations between lower GI tract symptomatology and the psychophysiological stress response (Ferguson et al., in press).

A significance level of 0.05 was used as a reference for interpreting results. No correction was made for multiple testing, and as such, conclusions may be interpreted as potential directions for future research. No differences were observed in any of the measures between the study sites. As such, the forthcoming statistics represent a combination of participants from both centers mentioned above.

Results

Gastrointestinal Symptoms. As previously reported in Chapter 2 of this dissertation, the most frequently occurring GI disorders present in the sample according to parental- and self-report on the QPGS were: functional constipation (42.5%), irritable bowel syndrome (11.7%), and lower abdominal pain associated with bowel symptoms (9.2%), and upper abdominal pain associated with bowel symptoms (7.5%). As such, our analyses focused on these GI disorders.

Cortisol. A significant positive relationship was found between cortisol response to stress and a greater lower GI tract score from the QPGS, $r = 0.27$, $p = 0.021$, 95% CI = [0.04–0.47], controlling for age, gender, and pre-stress cortisol concentration. The latter was uncorrelated with lower GI tract score and post-stress cortisol in this sample (Table 4.1).

Biomarker	Covariate	Correlation (95% CI)	p-Value	n
Cortisol Response	Upper GI Score	−0.00 (−0.24, 0.23)	0.9755	75
	Lower GI Score	0.27 (0.04, 0.47)	**0.0207**	75
	IQ	0.27 (0.02, 0.49)	**0.0365**	64
	ABC Inappropriate Speech	−0.27 (−0.47, −0.04)	**0.0231**	74
	ABC Hyperactivity	−0.28 (−0.48, −0.05)	**0.0186**	74
IL-6 Concentration	Upper GI Score	0.13 (−0.06, 0.31)	0.1910	110
	Lower GI Score	−0.01 (−0.20, 0.18)	0.9320	110
	IQ	−0.29 (−0.46, −0.08)	**0.0062**	92
	Vineland Socialization SS	−0.27 (−0.47, −0.05)	**0.0169**	77
TNF-α Concentration	Upper GI Score	0.20 (0.01, 0.38)	**0.0391**	108
	Lower GI Score	0.08 (−0.12, 0.26)	0.4430	108
	IQ	−0.06 (−0.26, 0.15)	0.6026	91
	ABC Irritability	0.20 (0.01, 0.38)	**0.0433**	105

Table 4.1. Partial Pearson correlations between biomarkers and QPGS scores, FSIQ, and selected ABC and Vineland variables, controlling for age, gender, and cortisol pre-stress values (cortisol response only). Significant correlations are in bold ($p < 0.05$).

Furthermore, cortisol response significantly modified the relationship between lower GI tract score and psychophysiological stress response as measured by change in pNN50 due to cold- pressor stimulation ($p = 0.040$ for the interaction term, slope between lower GI tract score and change in pNN50 at quartile 1 of cortisol response = 17.88, slope at quartile 3 = 36.87). The negative correlation between psychophysiological stress response and lower GI tract score increases as the endocrine stress response increases. Change in pNN50 due to cold-pressor stimulation is uncorrelated with cortisol concentrations in this sample, and both pNN50 stress response and cortisol response are significant independent predictors of lower GI tract symptoms.

Potential Effect Modifiers. Presence of regressive autism was found to be a significant effect modifier for the relationship between lower GI tract score and cortisol response ($p = 0.007$ for the interaction term, slope in the presence of regressive autism = 11.88, slope in the absence of regressive autism = 0.79) (Figure 4.1).

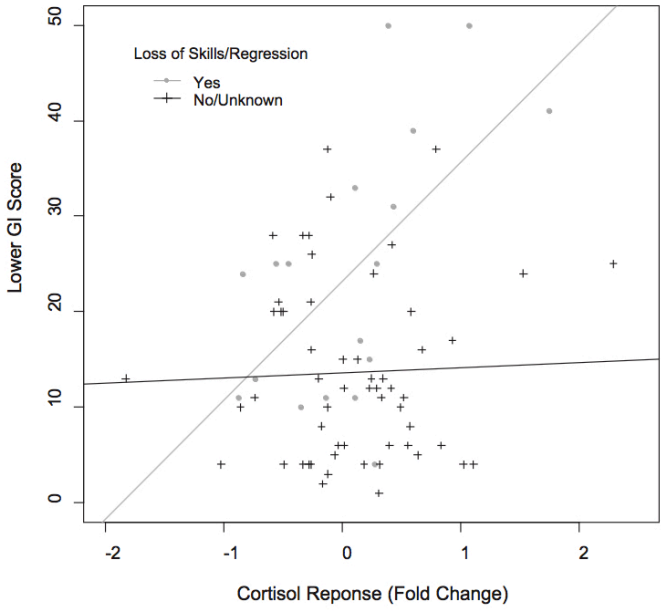

Figure 4.1. Illustration of the effect modification of history of regressive autism on the relationship between cortisol stress response and lower GI tract scores.

Exploratory Analyses. A significant positive correlation was identified between cortisol response to stress and FSIQ, $r = 0.27$, $p = 0.037$, 95% CI = [0.02– 0.49]. In contrast, significant negative correlations were found between the cortisol response to stress and both the ABC Inappropriate Speech subscale, $r = -0.27$, $p = 0.023$, 95% CI = [0.47 to 0.04] and the Hyperactivity subscale, $r = -0.28$, p = 0.019, 95% CI = [0.48 to 0.05] (Table 4.1).

IL-6 and TNF-α. For IL-6, no significant relationship was found with upper or lower GI tract scores from the QPGS. For TNF-α, there was a significant positive correlation between TNF-α concentration and the QPGS upper GI tract score while controlling for age and gender, $p = 0.039$, r = 0.20, 95% CI = [0.01–0.38] (Table 4.1). For IL-6 concentration, a significant negative relationship was found with FSIQ, $p <$ 0.01, $r = -0.29$, 95% CI= [0.46 to 0.08], and a significant negative relationship was found

with the Vineland Socialization domain scale score, $p = 0.017$, $r = 0.27$, 95% CI = [0.47 to 0.05] (Table 4.1).

For TNF-α concentration, a significant negative relationship was found with the ABC Irritability score, $p = 0.043$, $r = 0.20$, 95% CI = [0.01–0.38] (Table 4.1).

For key co-occurring conditions, a significant relationship was found between TNF-α concentration and presence of an anxiety disorder ($p = 0.037$, Cohen's $d = 0.423$) (Table 4.1). A weak relationship was observed between TNF-α concentration and reporting regressive autism ($p = 0.096$, Cohen's $d = 0.351$). In both cases, subjects with the condition had higher TNF-α concentrations on average.

No such relationships were observed with depression or seizures.

Discussion

To our knowledge, this is the first study to examine the interrelationships between GI symptoms in ASD and stress-related endocrine as well as immune markers associated with stress response. The present multi-site study found a significant positive relationship between symptoms of the lower GI tract and cortisol responses to stress. Cortisol stress-response also increases the negative relationship between symptoms of the lower GI tract and the physiological stress response measured by change in pNN50, suggesting that the relationship between psychophysiology and GI symptoms is associated with the stress response. The presence of regressive autism significantly modified the relationship between the cortisol response to stress and lower GI tract symptoms, such that among individuals with regressive ASD, lower GI tract disorders were strongly associated with an increased stress response. This has not been previously observed. Neuropathological findings in ASD include atypical amygdala structure and function (Nordahl et al., 2012),

and the amygdala is intimately involved in the response to stress. Further, myeloid dendritic cells, which produce IL-1B, IL-6, TNF-α, and IL-12, are associated with increased amygdalar volume, regressive autism, and increased GI symptoms in ASD (Breece et al., 2013). In the context of the finding from the present study, it appears that the interplay among the immune system, HPA axis reactivity, and stress-associated brain regions is important in relation to symptoms of the lower GI tract in ASD. Furthermore, developmental trajectories in ASD diverge, with regressive ASD being associated with heightened developmental disruption relative to those without regressive ASD (Landa et al., 2013). Future research should explore the implications of the immunological and stress response profiles of individuals with regressive ASD as compared to non-regressive ASD. It may be that the altered trajectory associated with regression itself contributes to these hormonal and stress reactivity changes. Regardless of the mechanism, this will also be important as it relates to general health, including GI functioning, to determine if individuals with regressive ASD are at a heightened risk for negative health outcomes.

The increases in cortisol response, however, do not appear to translate into effects on the stress-related cytokines, TNF-α and IL-6. The modest relationship between upper GI tract symptoms and TNF-α is consistent with previous findings of increased TNF- a associated with GI symptoms in general (Ashwood et al., 2011), but this is limited by the paucity of upper GI tract symptoms in our sample. For those previous studies, there was no separate analysis for subtypes of GI symptoms, but lower GI tract symptoms predominated.

Exploratory analyses revealed several associations between cortisol and immune

biomarkers, co-occurring psychiatric conditions, and intelligence. A robust negative relationship between intelligence and IL-6 emerged. Increased IL-6 concentration has been shown in individuals with ASD (Yang et al., 2015), and has been proposed to be an important factor in the etiology and severity of ASD-like behaviors (Wei, Alberts, & Li, 2013). Causal relationships are not able to be made from these correlational analyses, but our findings in the setting of this proposed role is of interest. The relationship between anxiety and TNF-α is not surprising given that the cytokines measured in this study are associated with the stress response in those without ASD. Both cortisol and heart rate-related responses have also previously been shown to be related to anxiety in ASD (Hollocks et al., 2014). The negative relationships between TNF-α and irritability and between IL-6 and sociability are consistent with previous observations of decreased immune markers with behavioral symptoms in ASD (Heuer et al., 2008). Alterations in other cytokines have been observed in ASD with a history of regressive autism (Lyte et al., 2011), but our study only found a weak association with TNF-α, among stress-associated cytokines. Cytokine activation, including TNF-α, is associated with major depression in those without ASD, in addition to HPA axis dysregulation (Dantzer et al., 2008), and seizure disorders are also associated with HPA axis dysregulation in those without ASD (Majoie et al., 2011). We found no relationships in our ASD population, which may be related to the low incidence of seizures and the low degree of depressive symptomatology in our study sample. Anxiety among those without ASD is also associated with significant alterations in the HPA axis and a range of cytokines (O'Donovan et al., 2010), and HPA axis changes are also observed in irritable bowel syndrome (Chang et al., 2009), which relate to our findings for ASD. While, as described

above, effects are known on stress reactivity and cytokines for anxiety, depression, and seizures in typically-developing individuals, it will be important in future studies to see how this differs in ASD, to see if different mechanisms might be present. This may help guide future treatments. Given the exploratory nature of the findings described above, though, they should be interpreted with caution.

The relationship between lower GI tract symptoms and stress-related endocrine response raises the possibility that amelioration of the stress response might be an important aspect of treatment in some cases. The relationship between lower GI tract symptoms and stress reactivity specifically among those with regressive autism needs replication but warrants further study, to understand its potential role in this poorly understood subgroup of ASD. The relationship between IL-6 and performance on intelligence tests is also of interest for future research given the previous work proposing a relationship between IL-6 and autism severity. However, despite the relationship between lower GI tract symptoms and stress-related endocrine markers, there was no relation- ship between stress-related cytokines and lower GI tract symptoms.

A potential limitation of the current study is the omission of individuals with ASD and mitochondrial disorders or genetic disorders such as Fragile X syndrome and tuberous sclerosis. As such, the current findings are not generalizable to these individuals. Additionally, with our sample size we targeted the specific cytokines most related to stress, ASD, and GI symptomatology. Larger studies in the future should explore a broader range of cytokines. Last, as mentioned in the Statistical Methods section, conclusions from the present study were based on significance levels uncorrected significance for multiple comparisons, in this exploratory study, and so the findings

herein should be investigated further in the future.

In summary, we found that greater lower GI tract symptoms were significantly associated with cortisol concentration after exposure to stress. This relationship between cortisol response to stress and GI functioning was greatest for children who had a history of regressive autism. Significant correlations were also observed between cortisol response, intelligence, and inappropriate speech. Lower GI tract symptoms were not associated, though, with levels of TNF-α or IL-6. However, significant relationships were observed between the cytokines and irritability, socialization, and intelligence. These findings suggest that individuals with ASD and lower GI tract symptoms may have an increased response to stress, but this effect is not associated with concomitant changes in stress-associated cytokines. The relationship between cortisol stress response and lower GI tract symptoms in children with regressive autism, as well as the relationships between cortisol, IL-6, and intelligence in ASD, warrants further investigation for potential implications for the mechanism as well as for potential impact on treatment.

CHAPTER FIVE:

OTHER RELATED RESEARCH TO WHICH WE CONTRIBUTED TO AS PART OF THIS COLLABORATIVE PROJECT WITH VANDERBILT UNIVERSITY: WHOLE BLOOD SEROTONIN LEVELS AND GASTROINTESTINAL SYMPTOMS IN AUTISM SPECTRUM DISORDER[1]

Introduction

Elevated whole blood serotonin (5-hydroxytryptamine, 5-HT), termed hyperserotonemia, occurs in approximately 30 % of children with ASD (Gabriele et al. 2014). Blood 5-HT is synthesized in the gut, where it plays a critical role in regulating motility and inflammation (Gershon, 2013). Deletion of enteric 5-HT neurons has been shown to greatly impair GI motility in rats (Piñeiro-Carrero et al., 1991), and so is possible that hyperserotonemia leads to desensitization of the GI tract, resulting in constipation. GI symptoms are more prevalent in children with ASD than in controls, with constipation most commonly reported (Aldinger et al. 2015; Chaidez et al. 2014; Chandler et al. 2013; Gorrindo et al. 2012; Ibrahim et al. 2009; McElhanon et al. 2014).

To our knowledge, no previous study examined the relationship between GI symptoms and 5-HT in ASD. Based upon the enteric 5-HT system's impact on gut motility (Gershon 2013; Li et al. 2011), we hypothesized that children with hyperserotonemia have reduced motility and resulting functional constipation. We also explored potential association with behavioral symptoms based upon previous, inconsistent findings (Kolevzon et al. 2010; McBride et al. 1998; Mulder et al. 2004; Sacco et al. 2010), as well

[1] Marler, S., Ferguson, B.J., Batey Lee, E., Peters, B., Williams, K., McDonnell, E. …Veenstra-VanderWeele, J. (2016). Brief report: Whole blood serotonin levels and gastrointestinal symptoms in autism spectrum disorder. *Journal of Autism and Developmental Disorders*, *46*, 1124-1130.

as with IL-6 levels following a recent report of correlation (Yang et al. 2015). Finally, we examined non-verbal status as a potential confounder based upon its previous association with functional constipation in ASD (Gorrindo et al. 2012).

Methods

Participants. This study was approved by the Institutional Review Boards at the University of Missouri and Vanderbilt University. Consents, and, when appropriate, assents, were obtained from participants and/or parents. As previously described in Chapter 2 of this dissertation, 120 individuals with ASD, aged six to eighteen, were recruited through the Autism Speaks - Autism Treatment Network and affiliated clinics. Parents of potential participants were screened via telephone to attempt to recruit a similar number of participants with and without a GI disorder. All participants were diagnosed with ASD based on the DSM-IV criteria (American Psychiatric Association 2000), using the Autism Diagnostic Observation Schedule (ADOS) (Lord et al. 1989) to verify. Thirty-eight participants were excluded because they were taking medications that affect 5-HT, including serotonin reuptake inhibitors, psychostimulants, and neuroleptics.

Assessment of Gastrointestinal Symptoms. As described in Chapter 2 of this dissertation, the QPGS (Walker, Caplan-Dover, & Rasquin-Weber, 2006) was used to assess GI symptoms. Parent-report forms were administered to most families. Standard criteria were used to assign functional GI diagnoses for categorical analyses (Walker, Caplan-Dover, & Rasquin-Weber, 2006). To allow the analysis of GI symptoms on a continuum and to assess relative severity of lower GI symptoms, the QPGS was also scored using a previously described scoring rubric described in Chapter 2 of this dissertation.

Behavioral Measures. Sensory symptoms, interfering behaviors, repetitive speech, and anxiety were assessed using caregiver-reported measures: Sensory Over-Responsivity Inventory (SensOR) (Schoen et al. 2008), Aberrant Behavior Checklist (ABC) (Aman 1994), and Repetitive Behavior Scale- Revised (RBS-R) (Bodfish et al., 2000). Participants were defined as non-verbal if fewer than five words were used in the ADOS (module 1, item A1) (Gorrindo et al. 2012).

Serotonin. Fasting whole blood 5-HT was measured by high-performance liquid chromatography, as previously described (Anderson et al. 1987; McBride et al. 1998). To identify a "hyperserotonemia" subgroup for categorical analyses, whole blood 5-HT levels were compared to previously published levels in controls performed within the same laboratory (McBride et al. 1998), corrected for race, age, and sex.

Cytokines. IL-6 was measured using R&D ELISA kits (Minneapolis, MN), in duplicate, quantified on a Spectramax M3 plate reader (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions.

Data Analysis. Categorical analyses were used to compare participants with hyperserotonemia versus normoserotonemia, functional constipation versus no GI diagnosis, and verbal versus non- verbal groups using Fisher's exact tests for categorical variables and one-way analysis of variance for quantitative variables. Since significant skewness was observed, 5-HT levels were log-transformed for quantitative analyses. Pearson's and Spearman's correlations were applied in the Caucasian-only population with correction for age and sex (McBride et al. 1998). All analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC) and figures created in R version 3.1.2.

Results

Comparing to published norms (McBride et al. 1998), 23% of the participants had 5-HT levels two standard deviations above the mean for their race and pubertal status. The distribution of whole blood 5-HT levels showed significant skewness, with an extended tail toward elevated levels (Fig. 5.1). Participants with hyperserotonemia did not differ significantly from the rest of the participants on demographic characteristics, ADOS severity, or IQ (Table 5.1).

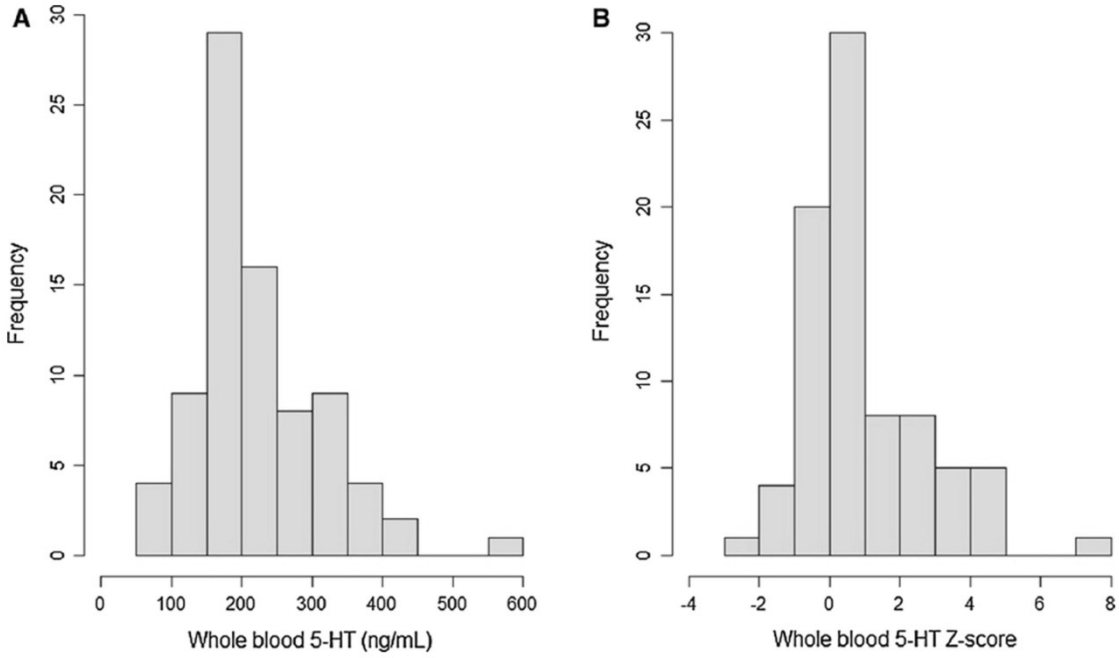

Figure 5.1. Distribution of whole blood serotonin (5-HT). Frequency distribution of 5-HT levels, in absolute values (ng/mL) (Panel A) and in Z scores (Panel B). 23% of the participants had 5-HT levels two standard deviations above the mean for their race and pubertal status.

	Total *N*	Normoserotonemia (*n* = 63)	Hyperserotonemia (*n* = 19)	*p* value
Sex	82			1.0
Male (%)	74 (90.2)	57 (90.5)	17 (89.5)	
Female (%)	8 (9.8)	6 (9.5)	2 (10.5)	
Age: Mean (SD)	82	11.2 (4.1)	11.7 (3.6)	0.61
Race	82			0.66
Caucasian (%)	75 (91.5)	58 (92.1)	17 (89.5)	
Other race (%)	7 (8.5)	5 (7.9)	2 (10.5)	
Ethnicity	82			1.0
Non Hispanic/Latino (%)	79 (96.3)	60 (95.2)	19 (100)	
Hispanic/Latino (%)	3 (3.7)	3 (4.8)	0 (0.0)	
Household income	71			0.58
Less than 50k (%)	31 (43.7)	25 (46.3)	6 (35.3)	
50k or greater (%)	40 (56.3)	29 (53.7)	11 (64.7)	
ADOS severity score: Mean (SD)	65	7.1 (2.3)	6.6 (2.0)	0.46
IQ: Mean (SD)	68	83.9 (24.4)	83.8 (21.0)	0.99

Table 5.1. Sample characteristics and relationship with 5-HT levels.

Functional constipation (FC) was the most common Rome III diagnosis, occurring in 39% of the sample. Participants with FC did not differ from those with no GI diagnosis on demographic characteristics, ASD severity, or IQ (Table 5.2). Lower GI symptoms were not significantly higher in the non-verbal subgroup (20.3 ± 15.6, n = 6) in comparison to the verbal subgroup (15.7 ± 11.2, p = 0.341).

	Total *N*	No GI diagnosis (*n* = 44)	Functional constipation (*n* = 32)	Non-retentive fecal incontinence (*n* = 2)	Other Rome-III diagnoses (*n* = 4)	*p* value
Sex	82					0.40
Male (%)	74 (90.2)	41 (93.2)	28 (87.5)	2 (100)	3 (75.0)	
Female (%)	8 (9.8)	3 (6.8)	4 (12.5)	0 (0)	1 (25.0)	
Age: Mean (SD)	82	12.0 (4.3)	10.6 (3.4)	10.0 (4.2)	10.8 (4.4)	0.41
Race	82					0.42
Caucasian (%)	75 (91.5)	38 (86.4)	31 (96.9)	2 (100)	4 (100)	
Other race (%)	7 (8.5)	6 (13.6)	1 (3.1)	0 (0)	0 (0)	
Ethnicity	82					0.66
Non-Hispanic/Latino (%)	79 (96.3)	43 (97.7)	30 (93.8)	2 (100)	4 (100)	
Hispanic/Latino (%)	3 (3.7)	1 (2.3)	2 (6.3)	0 (0)	0 (0.0)	
Household income	71					1.0
Less than 50k (%)	31 (43.7)	16 (42.1)	13 (44.8)	1 (50.0)	1 (50.0)	
50k or greater (%)	40 (56.3)	22 (57.9)	16 (55.2)	1 (50.0)	1 (50.0)	
ADOS severity score: Mean (SD)	65	7.1 (2.6)	6.7 (1.9)	7.5 (2.1)	7.0 (1.0)	0.86
Verbal status	73					1.00
Verbal	67	38 (90.5)	24 (92.3)	2 (100)	3 (100)	
Nonverbal	6	4 (9.5)	2 (7.7)	0 (0)	0 (0)	
IQ: Mean (SD)	68	85.9 (23.5)	80.8 (24.9)	72.0 (5.7)	88.3 (21.5)	0.71

Table 5.2. Sample characteristics and relationship with GI symptoms.

FC diagnosis was present in 10/19 (53%) of the hyperserotonemia subgroup and 22/57 (39%) of the remaining participants, excluding those with other GI diagnoses (Fisher's exact $p = 0.30$). As a quantitative measure, the QPGS lower GI score differentiated the FC (27.3 ± 10.0) and no GI disorder groups (8.3 ± 4.9, $p < 0.001$), but it did not differ in the hyperserotonemia subgroup in comparison to the rest of the participants. Approaching the analysis from the opposite perspective, normalized whole blood 5-HT levels were not significantly higher in the FC group (Mean $Z = 1.3 \pm 1.9$) in comparison to those with no GI diagnosis (Mean $Z = 0.7 \pm 1.6$, p = 0.14).

When considered as a continuous variable in the Caucasian- only sample, controlling for age and sex, a trend-level Pearson's correlation ($r = 0.21$, $p = 0.066$) and a marginally significant Spearman's correlation ($\rho = 0.23$, $p = 0.048$) were observed

between log-transformed whole blood 5-HT levels and the QPGS lower GI score (Figure 5.2). Exploratory, item-level Spearman's correlations were identified between whole blood 5-HT levels and pain around or below the belly button ($\rho = 0.36$, $p = 0.001$), resisting bowel movements/ "holding in" stool ($\rho = 0.30$, $p = 0.011$), and large bowel movements that clogged the toilet ($\rho = 0.25$, $p = 0.030$).

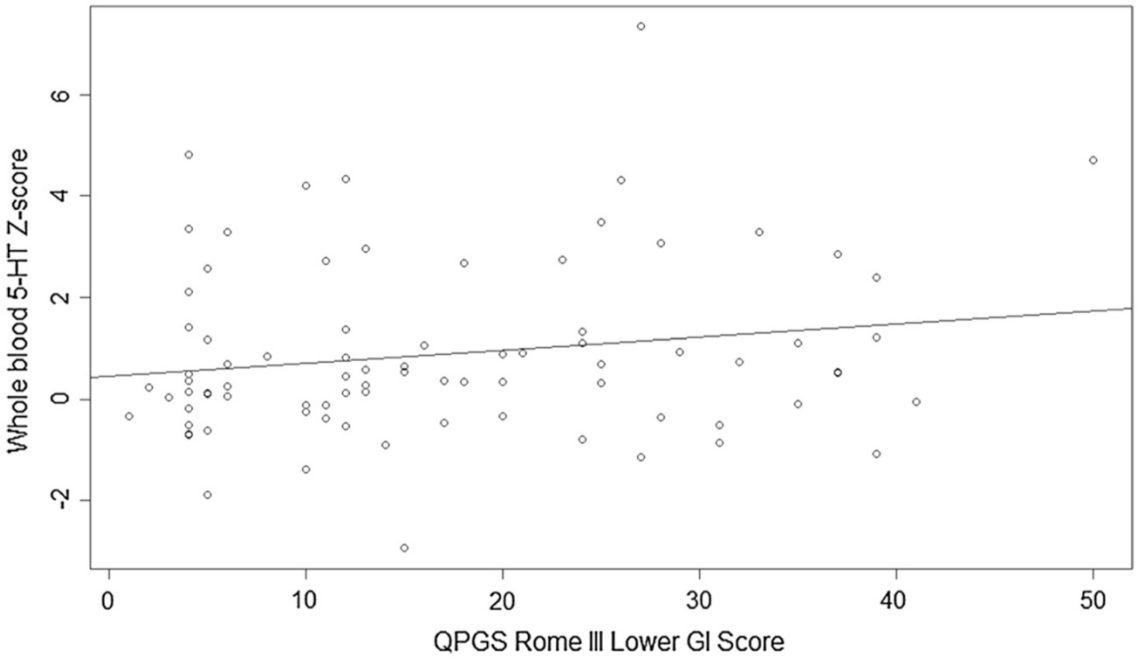

Figure 5.2. Correlation between QPGS lower GI scores and log-transformed whole blood serotonin raw scores. When considered as a continuous variable in the Caucasian-only sample, with age and sex as covariates, a trend-level Pearson's correlation (r = 0.21, p = 0.066) and a marginally significant Spearman's correlation (p = 0.23, p = 0.048) were observed.

The hyperserotonemia group did not differ significantly on any behavioral measure, nor did whole blood 5-HT levels correlate with these measures. A trend-level association was seen with RBS-R compulsive behavior, with higher scores in the hyperserotonemia subgroup (6.4 ± 6.5 vs. 4.1 ± 4.3, $p = 0.07$), without correction for multiple comparisons.

IL-6 levels did not differ significantly in the hyperserotonemia subgroup (1.6 ± 1.5 vs. 1.5 ± 0.9 pg/mL, $p = 0.783$), and no significant correlation was observed between IL-6 and whole blood 5-HT levels.

Discussion

The study population in the present study showed a significant rightward skew for whole blood 5-HT levels, with a similar proportion in the predicted hyperserotonemia range as in previous studies (Gabriele et al. 2014; McBride et al. 1998). FC was the most common GI diagnosis in our sample, which is also consistent with previous findings in ASD (Gorrindo et al. 2012). The lack of significant association between hyperserotonemia and FC suggests that there is not a unifying subgroup of children with ASD defined by elevated blood 5-HT levels and constipation. Further, these results do not support the hypothesis that constipation somehow leads to hyperserotonemia, or, conversely, that the mechanisms underlying hyperserotonemia also cause predictable GI symptoms. We cannot rule out a weaker relationship since the observed ratio was 1.8, which would require a four-fold larger sample to test.

Quantitative analyses are a more powerful way to detect association, and we did identify a correlation between whole blood 5-HT levels and lower GI symptoms. Further, we found correlations between 5-HT levels and three QPGS items that may indicate substantial constipation, but these are exploratory analyses that need to be confirmed in a larger, independent sample. These findings suggest that whole blood 5-HT levels may relate to GI symptoms, but they do not clarify whether this association is specific to ASD.

Consistent with previous efforts to identify symptom-level associations, we did not find any significant relationship between hyperserotonemia and behavioral outcomes.

We also failed to replicate the recent report of a correlation between whole blood 5-HT levels and IL-6 levels (Yang et al. 2015), with IL-6 similar to other reports (Ashwood et al. 2011; Masi et al. 2015). Finally, we did not observe the expected association between FC and minimally verbal status in this population, likely because our population only included six non-verbal children.

This study has a number of limitations. First, GI diagnoses were made based on parent report using the QPGS and not direct observation by a gastroenterologist or by examination of the stool. Direct stool assessment might provide a more valid, quantitative analysis, and would also allow examination of microbiota, which were recently shown to alter blood 5-HT levels in mice (Yano et al. 2015). Behavioral symptoms were similarly measured using well-validated parent report measures that still fall short of direct observation. As an additional concern, our sample size, while larger than most studies of this robust biomarker, is too small to detect moderate effect sizes in the subgroup of children with hyperserotonemia. Finally, a cross-sectional study without a treatment component does not allow us to make causal inferences regarding the observed, weak correlation.

In summary, our data confirm the frequency of hyperserotonemia and of FC in ASD, but there is no significant association between membership in these two subgroups. In contrast, analyses of these variables as continuous traits reveals a correlation of 5-HT levels with lower GI symptoms, including pain, stool retention, and large bowel movements. A longitudinal follow-up study with a larger sample size, including participants without ASD, would be necessary to understand whether these findings are specific to ASD and to clarify the directionality of this weak correlation.

CHAPTER SIX:

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Current prevalence estimates suggest that 1 in 68 children in the United States are diagnosed with ASD by eight years of age, with the disorder disproportionately affecting males at a rate of 4:1 (Centers for Disease Control and Prevention, 2014). Furthermore, estimates suggest that up to 90% of individuals with ASD suffer from co-occurring GI issues (Buie et al., 2010), those estimates vary widely. As previously stated in Chapter 1, a significant portion of individuals with ASD are non-verbal, and are unable to self-report GI symptoms. As such, problem behavior may develop because of an inability to verbally communicate abdominal pain (Buie et al., 2010). Problem behavior has been shown to be increased in individuals with ASD relative to their typically-developing siblings (Hovarth & Perman, 2002), which can often be predicted by and GI symptoms (Couray et al., 2012; Bishop-Fitzpatrick et al., 2015; Nikolov et al., 2009; Peters et al., 2014; Mazurek et al., 2013; Maenner et al., 2012). As such, most pharmacotherapeutic treatments in ASD address psychiatric manifestations. Current treatments for constipation in ASD often include laxatives, which are not effective in many patients. Given the association of GI disorders with problem behavior, it is imperative to find treatments to improve the quality of life for individuals with ASD and co-occurring GI disorders.

The combined results of the aforementioned studies in this dissertation suggest that alterations in the brain-gut axis seem to be characteristic of individuals with constipation

in ASD. That is, a complex interaction between ANS, immune, and endocrine systems create an environment within the body that promote constipation in ASD.

Indeed, several studies have shown altered HPA axis regulation in ASD. Vagal afferents inhibit HPA axis activity and function to reduce cortisol secretion (Bruno et al., 1989; Miao et al., 1997). As well, increases in cortisol production have been shown to be associated with decreases in cardiac vagal tone, providing a framework for understanding the findings from our study (Cacioppo et al., 1995; Gunnar et al., 1995). It follows that future research may wish to investigate pharmacological interventions affecting the stress response in ASD while observing the effects on GI functioning, especially constipation. Identifying biomarkers in ASD – causal as well as those to provide treatment for co-occurring conditions – is important for developing targeted treatments for individuals living with ASD (Beversdorf, 2016).

Future Directions

The aforementioned program of research presented in this dissertation suggests a positive association between the response to stress and lower GI tract symptoms, especially constipation, in ASD. Evidence for this statement comes from previous studies in our lab which found significant associations between psychophysiological and endocrine lower GI tract symptoms in 120 individuals with ASD. Furthermore, upon completion of the studies described in Chapters 2-5, we examined whether the stress response could predict the response to polyethylene glycol, a laxative commonly used in ASD, with the hypothesis being that if an association exists between lower GI symptoms and a heightened stress response, then a negative relationship would exist between a measure of stress reactivity and reported response to polyethylene glycol. Follow-up calls

were made to the caregiver of the participant with ASD to ascertain their impression of the child's GI functioning using a modified Clinical Global Impression – Polyethylene Glycol Improvement measure. Caregivers were asked, "Have there been improvements in constipation since beginning polyethylene glycol?", with the choice of answers ranging from 1 – "very much improved," 4 – "no change," to 7 – "very much worse." Preliminary analyses from a sample of 9 individuals taking polyethylene glycol for constipation indicated a significant negative relationship between CGI-I score for polyethylene glycol and the log transformed fold change in cortisol concentration (post stress – pre-stress cortisol concentration) during the study visit (r = -0.746, p = 0.013) suggesting that increases in the response to stress are associated with decreases in the response to polyethylene glycol.

Furthermore, many individuals with ASD have restrictive diets based on personal preferences, and some individuals try gluten- and casein-free diets to ameliorate ASD symptoms. Given this, it is possible that GI disorders in ASD may be the result of alterations in the child's diet and not because of an augmented stress response. To address this possibility, we examined potential relationships between GI symptoms and nutrient intake from a subset of individuals that participated in the aforementioned studies in Chapters 3-5 in this dissertation. Specifically, 75 individuals from the University of Missouri Thompson Center for Autism & Neurodevelopmental Disorders completed a food frequency questionnaire (FFQ) (Ritter-Gooder) in which the participant's caregiver estimated the participant's food intake over the past month. These data were analyzed for nutritional intake using the on-line, publicly available United States Department of Agriculture Food Composition Database that provides nutrient information for each food

on the FFQ. Nutrient information for each food item endorsed on the FFQ was obtained, and total nutrient intake for each participant was summed to yield monthly nutrient intake (Table 6.1). Correlations were calculated between each nutrient value and upper and lower GI tract scores calculated from the QPGS for each participant. Upper GI tract symptoms were significantly correlated with total dietary fiber intake and vitamin B6 intake; however, these relationships did not survive Bonferroni correction. There were no significant associations between lower GI tract symptoms and dietary intake (Table 6.2).

Taken together, these data provide preliminary evidence to suggest that although polyethylene glycol may help some individuals with ASD and constipation, others with an enhanced response to stress may not receive benefit. As well, it appears that dietary intake does not appear to be associated with GI symptoms in this sample. However, it should be noted that these data are preliminary and need replication prior to being interpreted as fact. Regardless, a promising line of research in the future may be the exploration of pharmacological interventions that block the stress response and their concomitant effects on constipation in ASD.

Potential Treatment of Lower Gastrointestinal Tract Disorders with the Non-specific β-adrenoceptor Antagonist Propranolol. GI motility is centrally controlled through the sympathetic and parasympathetic branches of the ANS as well as through β-adrenoceptors located in the smooth muscle of the GI tract. β-adrenoceptor agonism is associated with decreased GI motility (DiMarino & Cohen, 1982). Interestingly, some studies indicate an increased adrenergic response in ASD (Lake, Ziegler, & Murphy, 1977; Launay et al., 1987). Propranolol is a non-specific *β*-adrenoceptor antagonist which inhibits the action of epinephrine and norepinephrine both

centrally and peripherally. The three subtypes of β-adrenoceptors (β_1-, β_2-, and β_3) have affinity for E and NE and are functionally present in the smooth muscle of the human colon (Manara et al., 2000). Furthermore, study of human colonic smooth muscle tissue showed that propranolol has affinity for all three β-adrenoceptor subtypes *in vitro* (Manara et al., 2000). In healthy individuals, propranolol has been shown to increase peristaltic amplitude in the distal portion of the esophagus following administration of propranolol, indicating increased motility (Lyrenäs & Abrahamsson, 1986). As well, administration of propranolol lead to increased motility in a dose-dependent manner in the distal colon (Lyrenäs & Abrahamsson, 1986). Therefore, as activation of the β-adrenergic system is associated with decreased GI motility, and a heightened adrenergic response has been shown in ASD, it follows that the β-adrenoceptor antagonist propranolol may provide benefit to those with constipation in ASD. The findings from this research may reduce constipation in some individuals with ASD and provide increases in their quality of life.

Final Remarks

The research contained in this dissertation suggests that alterations in the response to stress are associated with constipation in ASD. Given that preliminary pilot data suggest that propylene glycol seems to help some individuals with ASD and co-occurring constipation but not all individuals, suggests a subset of individuals with ASD and constipation may benefit from treatments targeting the NE system. One such treatment may be the beta-adrenergic antagonist propranolol. Therefore, future research examining the effects of propranolol on constipation in ASD is warranted, which may eventually

improve the lives for a significant number of individuals with ASD and co-occurring constipation.

Table 3.2.

Scoring Rubric Generated from The Pediatric Gastrointestinal Symptoms – Rome III. Item selection based upon consensus among all authors.

Upper GI Items from Rome III (*Reverse Scored)	Question Text	Scoring Range
A1	In the last 2 months, how often did you have pain or an uncomfortable feeling in the upper abdomen *above the belly button*?	0-4
A5	For how long have you had pain or an uncomfortable feeling *above the belly button*? (options range from ≤1 month to ≥1 year)	1-5
B16	In the last year, how many times did you have an episode of severe intense pain around the belly button that lasted 2 hours or longer and made you stop everything that you were doing?	0-4
B16a	During the episode of severe intense pain, did you have any of the following?	
a	No appetite	0-1
b	Feeling sick to your stomach	0-1
c	Vomiting (throwing up)	0-1
d	Pale skin	0-1
e	Headache	0-1
f	Eyes sensitive to light	0-1
	In the last 2 months, how often did you:	
D1	Burp (belch) *again and again* without wanting to?	0-4
D4	Swallow or gulp extra air? (You might hear a clicking noise when you swallow.)	0-4
D5	IN THE PAST YEAR, how many times did you vomit (throw up) *again and again without stopping for 2 hours or longer*?	0-4
D5a	For how long have you had episodes of vomiting again and again without stopping?	1-5
D5b	Did you usually feel nausea when you vomited again and again without stopping?	0-1
D5c	Were you in good health for several weeks or longer between the episodes of vomiting again and again?	0-1
D6	In the past 2 months, how often did food come back up into your mouth after eating?	0-4
D6a	Does this usually happen less than an hour after you eat?	0-1

Lower GI Items from Rome III (*Reverse Scored)	Question Text	Scoring Range
	In the last 2 months, when you hurt or felt uncomfortable above the belly button, how often:	
A6*	Did the hurt or uncomfortable feeling get better after you had a poop?	0-4
A7	Were your poops softer and more mushy or watery than usual?	0-4
A8	Were your poops harder or lumpier than usual?	0-4
A9	Did you have more poops than usual?	0-4
A10	Did you have fewer poops than usual?	0-4
A11	Did you feel bloated in the belly?	0-4
B1	In the last 2 months, how often did you have a belly ache or pain *in the area around or below the belly button*?	0-4
B4	For how long have you had belly aches or pain *around or below the belly button*? (options range from ≤1 month to ≥1 year)	1-5
	In the last 2 months, when you had a belly ache or pain around or below the bellow button, how often:	
	Did it get better after having a poop?	
B5*	Were your poops softer and more mushy or watery than usual?	0-4
B6	Were your poops harder or lumpier than usual?	0-4
B7	Did you have more poops than usual?	0-4
B8	Did you have fewer poops than usual?	0-4
B9	Did you feel bloated in the belly?	0-4
B10		0-4
C1*	In the last 2 months, how often did you usually have poops? (options range from ≤2 times a week to >3 times a day)	1-5
	In the last 2 months, what was your poop usually like?	
C2*	In the last 2 months, did it hurt when you had a poop?	1-5
C3	In the last 2 months, did you have a poop that was so big that it clogged the toilet?	0-1
C8		0-1
C9	Some children hold in their poop even when there is a toilet they could use. They may do this by stiffening their bodies or crossing their legs. In the last 2 months, while at home, how often did you try to hold in a poop?	0-4
	Did a doctor or nurse ever examine you and say that you had a huge poop inside?	
C10	In the last 2 months, how often was your underwear stained or soiled with poop?	0-1
C11	When you stained or soiled underwear, how much was it stained or soiled? For how long have you stained or soiled your underwear?	0-5
C11a		1-3
C11b		1-5

	Mean	*SD*	N
Rome III GI Upper Score	4.64	5.51	75
Rome III GI Lower Score	20.28	13.48	75
Water (g)	16572.97	11460.77	75
Energy (kcal)	20488.04	10495.77	75
Protein (g)	1173.56	577.61	75
Total Lipid Fat (g)	839.27	471.00	75
Carbohydrate (By Difference; g)	2155.64	1280.66	75
Dietary Fiber (Total; g)	236.28	170.27	75
Sugars (Total; g)	903.46	766.05	75
Calcium (mg)	18807.42	16132.60	75
Iron (mg)	124.27	83.14	75
Magnesium (mg)	4071.2	2601.42	75
Phosphorous (mg)	21675.08	13352.86	75
Potassium (mg)	43572.57	26575.60	75
Sodium (mg)	26558.41	16553.72	75
Zinc (mg)	158.42	86.66	75
Vitamin C (Total Ascorbic Acid; mg)	1460.16	1742.43	75
Thiamin (mg)	17.15	9.36	75
Riboflavin (mg)	28.66	20.36	75
Niacin (mg)	267.65	135.11	75
Vitamin B6 (mg)	36.26	29.70	75
Folate (DFE; µg)	4106.64	3945.34	75
Vitamin B12 (µg)	79.59	52.50	75
Vitamin A (RAE; µg)	8888.35	7170.05	75
Vitamin A (IU)	69048.11	73514.22	75
Vitamin E (Alpha Tocopherol; mg)	110.16	79.69	75
Vitamin D2 + D3 (µg)	84.82	79.37	75
Vitamin D (IU)	3410.78	3262.99	75
Vitamin K (Phylloquinone; µg)	1109.33	1096.81	75
Total Saturated Fatty Acids (g)	283.16	168.10	75
Total Monounsaturated Fatty Acids (g)	308.11	191.03	75
Total Polyunsaturated Fatty Acids (g)	176.61	106.67	75
Total Trans Fatty Acids (g)	8.32	12.0	75
Cholesterol (mg)	3314.17	1822.61	75

Table 6.1. Mean nutrient intake values for foods reported on the FFQ for 75 participants from the University of Missouri Thompson Center.

	Rome III Upper GI Score	Rome III Lower GI Score
Rome III Upper GI Score	1	**.531
Rome III Lower GI Score	**.531	1
Water (g)	0.013	0.103
Energy (kcal)	-0.004	0.102
Protein (g)	-0.081	0.063
Total Lipid Fat (g)	-0.111	0.011
Carbohydrate (By Difference; g)	0.129	0.178
Dietary Fiber (Total; g)	*0.235	0.168
Sugars (Total; g)	0.224	0.132
Calcium (mg)	-0.055	0.085
Iron (mg)	0.088	0.088
Magnesium (mg)	-0.017	0.085
Phosphorous (mg)	-0.067	0.085
Potassium (mg)	0.01	0.117
Sodium (mg)	-0.028	0.071
Zinc (mg)	-0.002	0.106
Vitamin C (Total Ascorbic Acid; mg)	0.17	0.098
Thiamin (mg)	0.016	0.152
Riboflavin (mg)	-0.083	0.078
Niacin (mg)	-0.089	0.024
Vitamin B6 (mg)	**0.338	0.184
Folate (DFE; µg)	0.033	0.083
Vitamin B12 (µg)	-0.07	0.034
Vitamin A (RAE; µg)	-0.076	0.05
Vitamin A (IU)	0	0.056
Vitamin E (Alpha Tocopherol; mg)	-0.032	0.005
Vitamin D2 + D3 (µg)	-0.202	-0.043
Vitamin D (IU)	-0.203	-0.044
Vitamin K (Phylloquinone; µg)	0.107	0.106
Total Saturated Fatty Acids (g)	-0.073	0.029
Total Monounsaturated Fatty Acids (g)	-0.119	0.009
Total Polyunsaturated Fatty Acids (g)	-0.162	-0.031
Total Trans Fatty Acids (g)	-0.096	0.106
Cholesterol (mg)	-0.061	0.01

Table 6.2. Correlation matrix for the relationship between mean nutrient intake values for foods reported on the FFQ and upper and lower GI tract scores from the QPGS. ** $p < .01$; * $p < .05$. *p*-values shown are uncorrected.

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Bradley James Ferguson, son of Bill and Kathy Ferguson, was born and raised in Plano, Texas where he graduated from Plano East Senior High School in 1999. Bradley attended the University of North Texas in Denton, Texas, where he graduated with a Bachelor of Arts degree in Psychology in 2005. Bradley then moved to Murfreesboro, TN to attend Middle Tennessee State University where he earned a Master of Arts degree in Clinical Psychology with specializations in Clinical Neuropsychology and Applied Behavior Analysis in 2010. Upon completion of his master's degree, Bradley attended the University of Missouri in Columbia, Missouri, to study Neuroscience, focusing on biomarkers associated with gastrointestinal disorders individuals with autism spectrum disorder. Bradley graduated from the University of Missouri in December, 2016 with a Doctor of Philosophy degree in Neuroscience. He immediately began postdoctoral studies under the continued tutelage of Dr. David Beversdorf and other members of an interdisciplinary team. Bradley plans to continue studying pharmacological interventions on core symptomatology as well as co-occurring medical conditions in individuals with autism spectrum disorder.