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Associations between cytokines, endocrine stress response, and gastrointestinal symptoms in autism spectrum disorder

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Abstract

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Many children and adolescents with autism spectrum disorder (ASD) have significant gastrointestinal (GI) symptoms, but the etiology is currently unknown. Some individuals with ASD show altered reactivity to stress and altered immune markers relative to typically-developing individuals, particularly stress-responsive cytokines including tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6). Acute and chronic stress is associated with the onset and exacerbation of GI symptoms in those without ASD. The present study examined whether GI symptoms in ASD were associated with increases in cortisol, a stress-associated endocrine marker, and TNF- α and IL-6 in response to stress. As hypothesized, a greater amount of lower GI tract symptoms were significantly associated with post-stress cortisol concentration. The relationship between cortisol response to stress and GI functioning was greater for children who had a history of regressive autism. Exploratory analyses revealed significant correlations between cortisol response, intelligence, and inappropriate speech. In contrast, symptoms of the lower GI tract were not associated with levels of TNF- α or IL-6. Significant correlations were found, however, between TNF- α and IL-6 and irritability, socialization, and intelligence. These findings suggest that individuals with ASD and symptoms of the lower GI tract may have an increased response to stress, but this effect is not associated with concomitant changes in TNF- α and IL-6. 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, warrant further investigation.

Keywords

Autism spectrum disorder; Cortisol; Cytokines; Stress; Gastrointestinal disorders

1. Introduction

Autism spectrum disorder (ASD) is characterized by persistent deficits in social communication and restricted, repetitive patterns of behavior that occur early in development (American Psychiatric Association, 2000). Many studies suggest an increased prevalence of gastrointestinal (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 adrenocorticotrophic 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 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- γ , 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 et al., 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.

As a result, 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, and also 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.

2. Materials and methods

2.1. 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.

2.2. Assessment of gastrointestinal symptoms

The Questionnaire on Pediatric Gastrointestinal Symptoms-Rome III (QPGS-RIII) Walker et al., 2006 was used to assess GI symptomatology. The QPGS-RIII 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-RIII has been used to assess GI dysfunction in ASD with clinician-parent agreement at 92.1% for presence of any QPGS-RIII disorder, and fair agreement for functional constipation (Gorrindo et al., 2012). Consistent with previous work from our team, continuous variables were created for upper and lower GI tract symptoms (Ferguson et al., in press). Briefly, the multiple choice responses to the questions pertaining to the ten functional pediatric GI disorders assessed by the QPGS-RIII were assigned ratings, and a quantitative score was created by summing the ratings (scored on scales of 1–3, 0–4, 1–5, or 0–5, in accordance with the QPGS-RIII 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 that included the following GI disorders for each GI group: Upper GI – functional abdominal pain, abdominal migraine, aerophagia, upper abdominal pain associated with bowel symptoms; Lower GI – functional constipation, irritable bowel syndrome, non-retentive fecal incontinence, lower abdominal pain associated with bowel symptoms. Parent-report forms were used for all participants except for four individuals over the age of 16, as the parent indicated that the participant would provide the most accurate account of GI functioning.

2.3. 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).

2.4. Stress response protocol

All participants at each site engaged in a stress response protocol involving momentary, unpleasant physical stimuli, which has been shown to illicit a cortisol response in ASD (Taylor and Corbett, 2014). Cold pressor stimulation, a temporary stressor, was applied to one hand for 30 s at 4°C , followed by a 3-min break before applying the same stimulus and break period to the other hand (Zvan et al., 1998). Vibrotactile stimulation (Conair WM200X, Stamford, CT), a method to briefly increase heart rate (Foster et al., 2013), was also delivered to each hand with a 3-min break between each application. The order of cold pressor and vibrotactile stimulation along with the order of hand was counterbalanced across participants. Electrocardiogram (ECG) and skin conductance level data were collected from participants throughout the duration of the study. For the ECG data, motion artifacts were excluded, and R-R intervals were determined as previously described (Ferguson et al., in press; Tarvainen et al., 2014). Heart-rate variability, a measure of the balance of sympathetic and parasympathetic influences on the heart, was represented by the percent of successive normal R-R intervals that varied by 50 ms or more, referred to as pNN50. For the skin conductance data, records with excessive artifacts were excluded. Mean skin conductance level in microsiemens was calculated for the initial baseline reading and each vibrotactile and cold pressor condition.

2.5. 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).

2.6. 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.

2.7. Statistical methods

The quantitative measures of upper and lower GI tract functioning derived from the QPGS-RIII 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.

3. Results

3.1. Gastrointestinal symptoms

According to parental- and self-report on the QPGS-RIII, the most frequently occurring GI disorders present in the sample 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.

3.2. Cortisol

A significant positive relationship was found between cortisol response to stress and a greater lower GI tract score from the QPGS-RIII, $r = 0.27$, $p = 0.021$, 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. See Table 1.

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.

3.3. 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) (see Fig. 1).

3.4. 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 = $[-0.47 \text{ to } -0.04]$ and the Hyperactivity subscale, $r = -0.28$, $p = 0.019$, 95% CI = $[-0.48 \text{ to } -0.05]$. See Table 1.

3.5. IL-6 and TNF- α

For IL-6, no significant relationship was found with upper or lower GI tract scores from the QPGS-RIII. For TNF- α , there was a significant positive correlation between TNF- α concentration and the QPGS-RIII upper GI tract score while controlling for age and gender, $p = 0.039$, $r = 0.20$, 95% CI = $[0.01-0.38]$. See Table 1.

For IL-6 concentration, a significant negative relationship was found with FSIQ, $p < 0.01$, $r = -0.29$, 95% CI = $[-0.46 \text{ to } -0.08]$, and a significant negative relationship was found with the Vineland Socialization domain scale score, $p = 0.017$, $r = -0.27$, 95% CI = $[-0.47 \text{ to } 0.05]$. See Table 1.

For TNF- α concentration, a significant negative relationship was found with the ABC Irritability score, $p = 0.043$, $r = 0.20$, 95% CI = $[0.01-0.38]$. See Table 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 $d = 0.423$). See Table 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.

4. 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- α 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 et al., 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 is in need of replication but warrants further study, in order 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 relationship 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.

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Fig. 1. Illustration of the effect modification of history of regressive autism on the relationship between cortisol stress response and lower gastrointestinal tract scores.

Table 1

Partial Pearson correlations between biomarkers and QPGS Rome III GI 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 ($p < 0.05$).

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