Serum Zonulin Levels Are Higher Among Children with Autism Spectrum Disorders and Correlated with Social Impairment

ABSTRACT

Objective: Zonulin is a protein that affects the integrity of intercellular connections in the intestines. It has been emphasized that autoimmune diseases as well as neurodevelopmental disorders, for example autism spectrum disorder (ASD), may occur through alterations in intestinal permeability and blood-brain barrier. We aimed to investigate the gastrointestinal permeability of individuals with ASD by determining serum zonulin levels and their relationship to symptom severity.

Methods: Twenty-five ASD patients and 19 controls were included. Serum zonulin levels were measured by enzyme-linked immunosorbent assay kits. Clinical severity was assessed by the Childhood Autism Rating Scale (CARS), and social skills of the control group were evaluated by the Conners’ Parents and Teacher’s Rating Scales-Revised/Long Forms (CPRS-CTRS).

Results: Mean zonulin levels were significantly higher in the ASD group and positively correlated with CARS scores. After regression analysis, serum zonulin levels predicted CARS total scores. We could not find any significant correlation between zonulin levels and CPRS-CTRS sociability subscale scores in the control group.

Conclusion: The positive correlation between serum zonulin levels and ASD severity may require precaution for impaired intestinal permeability in clinical practice, especially for the cases in which sociability is severely impaired. However, it is too early to state that intestinal permeability has a role in the etiology of ASD. Further studies involving specific autism subgroups, and samples with certain dietary differences are needed.

Keywords: Autistic disorder, psychosocial functioning, haptoglobins, epithelium

Introduction

The fact that autism spectrum disorder (ASD) patients exhibit behavioral symptoms of many types and intensities makes the clinical appearance of ASD quite heterogeneous.1,2 Although its etiology has not been fully elucidated yet, twin studies have revealed that ASD is one of the most inherited disorders, with a genetic transition of up to 87%.3,4 It has been suggested that advanced paternal age, birth complications, and some environmental exposures in prenatal and neonatal periods also contribute to etiology.5

The effect of epigenetics on gene expression in ASD has become more interesting for clinicians, with numerous studies conducted in recent years. The potential role of the microbiota–gut–brain axis in pathogenesis is one of the focus of studies on this subject.6 Complaints related to the gastrointestinal (GI) tract occur in individuals with ASD approximately 5 times more frequently than in the general population, and cause disruption in areas such as sleep and behavior regulation; they may also lead to an increase in psychiatric comorbidity.7 Although the cause–effect relationship between them is not clear, it is claimed that these GI problems may be a condition supporting the connection between the gut–brain axis and neurodevelopmental disorders.8 In addition, findings obtained from some research studies...
that the microbiota are effective on central nervous system development and plasticity in the early postpartum period are in line with the data that intestinal flora affects the formation and symptom severity of neurodevelopmental disorders such as ASD.9

The intestinal epithelium is the longest mucosal barrier between the outside world and the human body. This anatomically and functionally special epithelium also regulates the traffic between the lumen and the intracellular environment. The passage of molecules is controlled by tight junctions between epithelial cells. It is now known that alteration of the intestinal flora in various ways activates some pathways, causing tight connections between the cells to relax, and the relaxed epithelium—that is, the "leaky gut"—somehow contributes to the etiology of autoimmune diseases by increasing the antigen exposure of many organs.10 In recent years, it has been emphasized that autoimmune diseases as well as neurodevelopmental conditions such as schizophrenia, attention deficit hyperactivity disorder (ADHD), and ASD may occur through this mechanism via alterations in the blood-brain barrier.11

Zonulin, the precursor of haptoglobin (HP-2), is a protein that affects the integrity of tight intercellular connections and its structure is similar to some growth factors. Intestinal tissue exposed to enteric bacteria and gladin interacts with CXCR3 chemokine receptors to release zonulin.12,13 Serum zonulin levels were detected to be higher in celiac cases than in healthy individuals.14 Zonulin has also been reported to be related to the development of neuropsychiatric disorders such as chronic inflammatory demyelinating polyneuropathy, multiple sclerosis, and schizophrenia.15 Autoimmune diseases bringing increased risk of ASD, the high frequency of GI symptoms in ASD patients, and some studies indicating the increased levels of zonulin in ASD, may suggest that alterations in the blood-brain barrier and intestinal epithelium may be causing ASD.16,17

Studying intestinal permeability in ASD with large samples and in a single center poses difficulties in terms of both funding and access to the patient group. Performing similar studies on the same subject at different centers and at different times is important in terms of creating a more objective data source. Our study aimed to support the literature on this issue. Besides, the use of only quantitative methods fail to capture perspectives from broad and complex groups. Thus, the relationship between autism and intestinal permeability has been studied quantitatively, adhering to theory-based, structured, systematic, planned, objective, logical, and scientific thinking.

**MAIN POINTS**

- Increased zonulin in the serum can show diminished integrity in the bowel mucosa, however zonulin examine in the faeces will ensure us to achieve a more specific result.
- Increased zonulin level in the blood may be related to the phenotype of ASD rather than the cause of ASD.
- Even if there is no direct relationship between ASD and intestinal permeability, perhaps intestinal permeability may facilitate an environmental disadvantage that influences common variable genes involved in ASD etiology.
- We think that, result of the study could lead to the evaluation that markers related to intestinal permeability could be used to diagnose with ASD, but for a specific group of those who have suffered more from GI symptoms.

We aimed to study the intestinal permeability of individuals with ASD by determining serum zonulin levels, and the relationship between these levels and symptom severity.

**Methods**

**Study Sample**

Cases were recruited from the outpatient clinic for child and adolescent psychiatry at the Aksaray University Training and Research Hospital. Children who were diagnosed with ASD, had a disability report, and received special education were evaluated as the ASD group. Exclusion criteria for the ASD group were determined as the presence of a major medical condition (such as diabetes mellitus, obesity, diagnosed GI disease), allergic, or neurological (such as epilepsy) diseases diagnosed as additional genetic syndromes related with ASDs, comorbidity of intellectual disability (ID was controlled clinically), body mass index (BMI) above 95%, intake of corticosteroids or any drugs that affect the immunological system at any time, and history of an infection within the last month. In particular, cases who had received any psychotropic drug therapy during the preceding 6 months were excluded from the study. ADHD comorbidity was evaluated with clinical examination and family interviews. Patients who had a suspicious history or a clinical finding related to ADHD were excluded. The control group was recruited from the outpatient clinic for pediatrics at the same hospital. After a pediatric examination, a psychiatrist screened the controls who were older than 6 years of age for psychiatric disorders, using the Schedule for Affective Disorders and Schizophrenia for School-Aged Children-Present and Lifetime Version-DSM-5 (K-SADS-PL-DSM-5).18,19 Two individuals in the control group under 6 years of age were clinically evaluated according to the DSM-5. When selecting the control group, the same exclusion criteria as for the case group were used, while the following were also added: presence of psychiatric disorders such as ID, ASD, schizophrenia, bipolar disorder, major depression, obsessive-compulsive disorder, and anxiety disorders. The study was reviewed and approved by the Ankara City Training and Research Hospital Ethical Committee (Number: E1/777/2020, Date: July 7, 2020). The subjects and their parents were informed about the purpose of the study, both verbally and in writing.

**Study Procedures and Measures**

Sociodemographic and clinical information were noted on a form prepared by the authors. BMI values were calculated manually for each subject. A child and adolescent psychiatrist evaluated patients and the controls for any psychiatric condition. The clinical severity of the ASD symptoms was assessed by the Childhood Autism Rating Scale (CARS). CARS has been found to be valid and reliable for the Turkish population.20 It consists of 14 domains evaluating the behaviors associated with autism, and a 15-domain scale rates the general impression of autism. Each of them is scored on a scale ranging from 1 to 4, higher scores being associated with more severe impairment. Total scores can range from 15 to 60, and scores below 30 indicate that the individual is not in the autistic spectrum. While the scores between 30 and 36.5 refer to mild to moderate autism, scores from 37 to 60 indicate symptoms of severe autism. The psychometrics of the CARS have been well documented.21

Social skills of the control group were evaluated with the Conners’ Teachers Rating Scale-Revised/Long Form (CTRS-R/L) and the Conners’ Parents Rating Scale-Revised/Long Form (CPRS-R/L). The
CPRS/R/L consists of these subscales: oppositionality, perfectionism, hyperactivity, cognitive problems/inattention, social problems, anxiety-shyness, and psychosomatic symptoms. Parents were told to complete the scales considering only the past month. It was answered as a 4-point Likert-type scale, answers ranging between “completely correct” and “not true at all.” The Turkish validity and reliability study was performed by Kaner et al. The scale will be briefly referred to as CPRS in the text.

The CTRS-R/L includes 38 items, 6 subscales, and 3 assistant scales based on the ADHD symptoms in ADHD. Teachers were asked to evaluate children’s behavior, considering only the last month during which they were with the children. For each item, the teachers answered in a 4-point Likert-type scale, the answers ranging between “completely correct” and “not true at all.” The Turkish validity and reliability study was performed by Kaner et al. The name of the scale will be briefly referred to as CTRS in the text.

Biochemical Analysis
Blood specimens were collected between 8.00 AM and 10.30 AM, after overnight fasting. They were centrifuged at 4000 rpm for 5 min at 4°C. When separated, sera were stored at −80°C until the assay time. Zonulin levels were assessed using commercial enzyme-linked immunosorbent assay kits following the manufacturer’s guidelines (Serum Zonulin, Sunred Biological Technology Co., Ltd, China; Cat No: 201-12-5578; Lot: 202003). The results were presented in ng/dL. Intra and inter-assay coefficients of variation of the kit were < 10% for both.

Statistical Analysis
Power analysis was done by G*Power software 3.1.9.6. (Franz Faul, Universität Kiel, Germany). Group sample sizes of 21 achieve 81% power to detect a difference of 35% for the mean levels of zonulin (ng/dL) between ASD and control groups, with a significance level (alpha) of 0.05 using a 2-sided 2-sample t-test. All other statistical computations, comparisons, and analyses were performed using SPSS for Windows, version 23.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to test normality. The factors that could correlate with the outcome (pulse–loss ratio) were analyzed independently (univariate analysis) by either Student’s t-test or Mann–Whitney U-test, where applicable. The differences in proportions between groups were compared by using the chi-square or Fisher’s exact test, where appropriate. Descriptive statistics were used to summarize the data and expressed as mean (standard deviation) for normally distributed continuous variables, median (minimum-maximum) for skewed continuous variables, and count with percentage of total for categorical variables. Degree of association between variables was calculated by Spearman correlation coefficients and summarized by using rho and the corresponding P values. Multiple linear regression equations were formulated to identify variables such as age, gender, and zonulin levels, which are independent predictor variables for the CARS scale total score. Assumption of linearity of the multiple regression model was tested by analysis of variance and corresponding statistics such as F and df were specified with the relevant P value.

Results
Twenty-five ASD (18 boys and 7 girls) and 19 (10 boys and 9 girls) healthy children were included. The mean age of the ASD group was 72.12 (SD = 33.68) months, and the mean age in the control group was 90.10 (SD = 29.31) months. There were no significant differences in terms of age between the ASD group and controls (P = .071). No significant difference was found between the controls and the ASD patients in terms of sex (P = .186). There was no significant difference between the BMI percentiles of the ASD patients and controls (P = .868). There were no significant differences in other demographic features either. The groups’ demographic and clinical variables summarized in Table 1.

When compared between the ASD and control groups, the mean zonulin level in the ASD group was 24.67 (SD = 19.42), and it was 14.45 (SD = 9.87) in the control group; there was statistically significant difference between the groups (P = .004) (Table 1).

Statistically significant correlation was found between zonulin levels and CARS total scores in the ASD group (r = 0.762, P < .001) (Figure 1).

With respect to subscales, a significant positive correlation was found between the zonulin levels and impaired verbal communication (r = 0.808; P < .001), impaired nonverbal communication (r = 0.790, P < .001), effectiveness level (r = 0.652; P < .001), listening (r = 0.687; P < .001), fear and irritability (r = 0.500; P = .011), adaptation to alteration (r = 0.416; P = .039), use of objects (r = 0.61; P < .001), use of body (r = 0.540; P < .001), emotional reactions (r = 0.618; P = .001), and human relationships (r = 0.694; P < .001).

In this context, it can be said that the level of zonulin level can predict the increase in CARS total scores. For this purpose, linear regression analysis was applied. CARS total scores were listed as dependent variables and age, gender and zonulin levels were listed as independent variables. Zonulin levels predicted CARS scores in a statistically significant way (β = 393, t = 4.899, P < .001). This model explained a significant proportion of variance in the CARS total scores (R² = 0.478, F = 8.315, P < .001) (Table 2).

Table 1. Demographic and Clinical Characteristics of Groups

|                      | Control (n = 19) | ASD (n = 25) | P     |
|----------------------|----------------|-------------|-------|
|                      | Mean (SD)      | Median (Min-Max) | Mean (SD) | Median (Min-Max) |       |
| Zonulin (ng/dL)      | 14.45 (9.87)   | 10.66 (4.22-44.11) | 24.67 (19.42) | 18.56 (7.35-69.67) | .004  |
| Age (months)         | 90.11 (29.31)  | 80.00 (60.00-163.00) | 72.12 (33.68) | 65.00 (28.00-145.00) | .071  |
| BMI                  | 21.99 (2.47)   | 21.55 (17.32-26.54) | 21.89 (1.77)  | 22.15 (17.53-27.12) | .868a |
| Sex (boy/girl)       | 10/9 to (52.6/47.4)% | 18/7 to (72.0/48.0)% | .186b |

aP values from Student’s t-test and others from Mann–Whitney U-test.

bP value for chi-square test.

BMI, body-mass index; ASD, autism spectrum disorder.
A separate evaluation on the CTRS and the CPRS indicated whether there was a correlation between social skills and zonulin levels of the control group. There was no correlation between social skill scores obtained in the CTRS and zonulin levels \((r = 0.149, P = .542)\). Similarly, there was no correlation between social skills scores scored in the CPRS and zonulin levels \((r = -0.031, P = .901)\).

**Discussion**

It is known that GI complaints are more common than normal in ASD patients.\(^{24}\) In fact, in a study, it was stated that GI symptoms are seen more in severe ASD cases than in mild cases.\(^{25}\) These data have increased the interest that there may be a relationship between ASD and gastrointestinal system (GIS). If we look at the literature published to date, we realize that when comparing high serum zonulin levels and physiological effects of zonulin with phenotypic evaluations of diseases, it proposes a model that is used to indicate the severity of diseases rather than being diagnostic.\(^{26}\)

In this study, in which we investigated the relationship between serum zonulin levels and ASD severity and socialization skills, we noticed that ASD symptoms were more severe as serum zonulin levels increased in the ASD group, but this increase was not observed in the healthy control group. Our study is one of the few studies on this subject.

Zonulin is a prehaptoglobulin that strengthens the connection between epithelial cells in the gut and influences intestinal permeability. Various environmental factors such as exposure to bacteria, and gluten and gliadin in the intestine may disrupt the structure of the “zonula occludens,” resulting in the zonulin being released into the serum. Opening of the paracellular pathway triggered by zonulin increase is evaluated as a protective mechanism, which flushes out microorganisms, thereby contributing to the innate immune response to these external stimuli.\(^{15}\) It can be thought that zonulin passing into the serum may influence the pathogenesis of ASD by triggering inflammation.\(^{27}\) In addition to low-grade inflammation, it is thought that some metabolites such as short-chain fatty acids that cross the blood-brain barrier may be effective in the pathogenesis of ASD by affecting mechanisms such as neural signals and neurotransmitter production.\(^{27}\) In a study, the presence of zonulin in the blood-brain barrier was associated with processes related with migration of neural stem cells to injury sites.\(^{28}\)

In one study, no statistically significant association was found between haptoglobin genotype, serum zonulin levels, and ASD.\(^{29}\) The same study compared zonulin levels in newborns, individuals with ASD, and healthy groups. In these studies, it was stated that serum zonulin levels were extremely high in children with ASD who had GI symptoms, but it was also stated that more studies are needed. They emphasized that it is necessary to explain the zonulin mechanisms in ASD in the larger population where detailed medical history and genetic characteristics were examined together. Like our study, many similar studies with smaller samples can also provide a database to elucidate the zonulin-ASD relationship.\(^{29}\) According to this study, low zonulin levels in healthy children and high zonulin levels in individuals with “some” ASD suggest that the zonulin level may be clinically important in “some ASD” cases. Studies conducted with newborns could not give an idea whether the increased zonulin levels at young ages cause the disease. However, they couldn’t explain the suspiciously high zonulin levels they encountered in the ASD group, and stated that studies with larger participation were needed. In our study, a very low level of significant difference was obtained between autism and the healthy group. However, this difference could not be obtained in a group with a larger sample. In this context, the results of Garcia’s study\(^{29}\) coincide with the results of our study.

**Table 2. Results of Multiple Linear Regression Analysis**

|       | B     | Std. Error | t     | P    | 95% CI Lower | 95% CI Upper |
|-------|-------|------------|-------|------|--------------|--------------|
| Constant | 26.134 | 6.700      | 3.901 | .001 | 12.202       | 40.067       |
| Age    | 0.054 | 0.045      | 1.194 | .246 | -0.040       | 0.148        |
| Sex    | -1.935| 3.115      | -0.621| .541 | -8.412       | 4.543        |
| Zonulin| 0.393 | 0.080      | 4.899 | <.001 | 0.226        | 0.560        |

B, regression coefficient \(P\) value for the regression model was found to be .001 \(F = 8.315, df = 3\).
However, it is known that immunity-related conditions such as autoimmune diseases, allergies, and psoriasis are more common in ASD.30 We know that immunity-related GI diseases (celiac, etc.) also increase in ASD.31 The fact that zonulin levels were found to be high in many diseases with GI involvement, in which the immune system is blamed in the pathogenesis, has led to discussion on whether the increased amount of zonulin has a place in the pathogenesis for autism symptoms in ASD patients with high GI symptoms.32 There are several studies investigating the association between ASD and intestinal permeability.33 Studies on this subject aim to reveal a new perspective regarding the etiology of autism and new options in treatment, with early diagnostic testing for ASD, especially while there are many rumors that the pathologies in the brain and gut axis are involved in the etiology of neurodevelopmental disorders.34 In the study of Esnafoglu et al.,17 serum zonulin levels of ASD patients were found to be higher than healthy controls. It was suggested that zonulin could contribute as an etiological factor to the development of ASD symptomatology via altering intestinal permeability.35 In addition, an editorial comment following the article mentioned was published in the same journal. In this letter, the author mentions that intestinal permeability is a topic discussed in ASD pathogenesis and it would be inappropriate to blame it in ASD pathogenesis.36 It was also emphasized that there could be a ambiguous relationship between the brain and gut. It was also suggested that loss of gut barrier function may not be generalized, and could be a phenomenon among a certain subgroup of ASD. For this reason, studies with larger samples will be needed.

Another point to consider is that intestinal permeability creates a problem that can affect the severity of autism rather than being present in the pathogenesis of autism, or this is only a concomitant problem that may have no relation with autism in severe cases. Therefore, there is a need for extensive studies on both the increase of intestinal permeability and the effects of increased intestinal permeability on neurons.

Pre-haptoglobin-2 (zonulin) is encoded by the haptoglobin (HP) allele-2 gene and provides tight intercellular junctions.37 Therefore, it can be thought that ASD is more common in cases where there is a defect in the gene encoding the zonulin protein. Contrary to popular belief, in a genetic study conducted in this subject with a large sample, the distribution of HP-2 gene alleles of patients diagnosed with autism and healthy controls was compared and no association was found between HP-2 and ASD. It had also been reported that the presence of GI disorders does not make a difference in terms of allele associations in the ASD group. Authors suggested that further investigations with wider sample and control groups are needed regarding this issue.38

In addition to these studies, Özyurt et al.11 found increased zonulin levels among ADHD patients and reported that these children have more social dysfunction than healthy controls. Moreover, serum zonulin levels were found to be an independent predictor for hyperactivity and social deficit scores in regression analysis.11 Similarly, in our study, we found that the serum zonulin levels can predict the increase in ASD severity. This result suggests that as the intestinal permeability increases, there may be more damage to ASD-associated areas in the brain.

However, when we evaluated specific areas of ASD functionality (CARS parameters: impaired verbal communication, impaired nonverbal communication, effectiveness level, listening, fear and irritability, adaptation to alteration, use of objects, use of body, emotional reactions, mimics, human relationships), we found that increased zonulin levels were associated with impairment in these areas. Therefore, we can assume that the impairment of intestinal permeability does not have a role in the pathogenesis of ASD but may lead to a change in the ASD phenotype. Even if there is no direct relationship between ASD and intestinal permeability, perhaps intestinal permeability may facilitate an environmental disadvantage that influences common variable genes involved in ASD etiology. This idea may explain why some children with ASD benefit from certain dietary recommendations. However, this idea is a proposition that must be studied.

Although the relationship between the gut–brain axis, microbiota, and ASD is a subject that has been studied frequently in recent years, treatment options which have been developed based on this hypothesis, such as diet, and use of prebiotics and probiotics, are evaluated as preliminary.34 Studies about this subject have some limitations and features that prevent generalizability—such as small sample sizes, placebo effect and selection bias, heterogeneity of ASD, and non-reproducibility of results obtained in animal models in humans.34 However, we cannot say whether impaired intestinal permeability is a cause for or a result of ASD. In a study about GI disorders and ASD association, 4 groups of children were included as follows: children with both autism and GI symptoms like inconsistency in bowel habits, children with autism without GI symptoms, and typically developing children with GI symptoms and those without GI symptoms.35 This study suggested that children with ASD and GI symptoms may have an instability in their immunity. In addition, there was no significant difference between children with ASD and typically developing groups in terms of HP2 over-representation, but there was a significant difference between children with ASD and GI symptoms and typically developing children with GI symptoms.36 We think this result could lead to the evaluation that markers related to intestinal permeability (i.e., zonulin, HP-2 alleles) could be used to diagnose with ASD, but for a specific group of those who have suffered more from GI symptoms. Nevertheless, although there is no sufficient marker for the diagnosis of all ASD patients, it can be a determinant in the severity of the disease. Perhaps, it may be possible to define a subgroup with GI symptoms later in ASD.

When we analyzed CTRS and CPRS social impairment scores in the healthy group for indications of whether serum zonulin levels might have affected social function, we did not find a relationship. This supports the idea that the intestinal-borne pathogens we mentioned earlier may have an epigenetic difference on the genes that contribute to the development of autism, because this increased intestinal permeability does not cause social problems in individuals without ASD susceptibility genes. Therefore, more studies are needed in order to say that zonulin has an effect on social functions.

Our study has certain limitations. First, our sample size is relatively small. We did not standardize our patients’ dietary habits, so dietary differences between patient and control groups could have influenced our results. This limitation is valid for most studies investigating gut–brain axis and ASD association. Second, zonulin acts as a tight junction connecting epithelial cells. It is found in the small intestinal epithelium, bladder epithelium, and between the blood vessel endothelium, in which cells form a barrier. In addition, there is a zonula occludens type connection in the blood–testis, blood–brain,
and blood–thymus barriers. Therefore, zonulin can pass into the serum from organs such as the lungs and the brain. However, if we were to study zonulin in stool, we could evaluate the level of zonulin specific to the intestine more accurately. Third, we realized that the study was necessary, with the division of the ASD group participating in the study into those with and without GI symptoms. Finally, our study was cross-sectional, and the patients were not followed up.

As a result, the increase in serum zonulin levels in the ASD group may not give the same result when studied with larger samples. However, in similar studies, a partial increase was frequently shown in zonulin levels compared to the control groups. This increase is likely to be related to ASD etiology, but it is not certain. Impaired intestinal permeability can cause damage to genes associated with ASD, or one of the gene loci in chromosome 16 that can be affected in ASD may be the HP-2 gene locus. Increased zonulin levels appear to be more related to the severity of ASD rather than its etiology. It is necessary to identify autism subgroups accompanied by GI symptoms and to conduct similar studies with them. More comprehensive studies are needed that include samples with specific nutritional differences. Further studies involving specific autism subgroups and samples with certain dietary differences are needed.

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