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Serum Zonulin and Claudin-5 but not Interferon-Gamma and Interleukin-17A Levels Increased in Children with Specific Learning Disorder: A Case-Control Study

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ABSTRACT

Background: Gut-blood and blood-brain barrier permeabilty (gut-brain axis) has been attracting increased attention in the etiology of neurodevelopmental disorders. In this study, we aimed to investigate serum levels of zonulin (a biomarker of intestinal permeability), claudin-5 (a biomarker of blood-brain barrier permeability), and interferon-gamma and interleukin-17A in children with specific learning disorder.

Methods: Forty-three children with DSM-5 diagnosis of specific learning disorder and 43 healthy children were included in this study. Serum levels of zonulin, claudin-5, interferon-gamma, and interleukin-17A were measured using commercial enzyme-linked immunosorbent assay kits.

Results: Serum zonulin and claudin-5 levels of the study group were significantly higher than the control group according to the multivariate analysis of covariance test while controlling for age, gender, and body mass index. However, serum interferon-gamma and interleukin-17A levels were not significantly different between the two groups. There was no correlation either between zonulin and interferon-gamma and interleukin-17A or claudin-5 and interferon-gamma and interleukin-17A.

Conclusion: Gut-blood and blood-brain barrier permeability may be disrupted in subjects with special learning disorder. Further research is needed to determine whether zonulin and claudin-5 may be biomarkers, and some dietary interventions or specific agents such as zonulin or claudin-5 inhibitors could be used in the management of neurodevelopmental disorders including special learning disorder.

ARTICLE HISTORY

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INTRODUCTION

Specific learning disorder (SLD) is classified within the neurodevelopmental spectrum, and its hallmark feature is the notable hindrance in acquiring and utilizing academic skills.[1] Specific learning disorder not only impairs children's ability to learn academic skills but is also associated with many undesirable conditions, such as low self-esteem, depression, anxiety, and behavioral problems.[2] A complete understanding of the cause of SLD has not been established yet. However, it is known that the interaction of genetic, epigenetic, and environmental factors affects brain structure and functioning.[3]

Current research has placed emphasis on the role of changes in the permeability of the intestinal epithelial barrier and the blood-brain barrier (BBB) in contributing to the development of neurodevelopmental disorders, as indicated by recent studies.[4,5] The gut-brain axis serves as a means of communication between the brain and the gut. Gut microbiota might affect neuroendocrine and neuroimmune interactions, the autonomic nervous system and brain function through the production of microbial toxins. The gut-brain axis has been implicated in the pathogenesis of neurodevelopmental disorders like attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) based on new findings.[6] The intestinal epithelial barrier formed by intestinal epithelial cells prevents the uncontrolled passage of ions, dissolved molecules, nutrients, microorganisms, and products of microorganisms from the intestinal lumen into the blood.[7] Epithelial passage occurs paracellular under the supervision of the apical junction complex formed by tight junctions (TJ).[8] Tight junctions, the apical component of intercellular junction complexes,

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provide structural integrity to endothelial and epithelial tissues. The alterations in intestinal permeability are associated with neuroinflammation and cerebrovascular permeability and are thought to cause neuropsychiatric manifestations and various chronic intestinal diseases.[7,9,10] Meanwhile the gut–brain axis is regulated by several protein biomarkers such as zonulin and claudins.[11,12]

Zonulin is the most important physiological modulator affecting intercellular TJ.[13] The main regulator of the permeability of both the gut-blood and BBBs is believed to be zonulin, which is utilized as a clinical marker for gut permeability.[14] There is a positive correlation between elevated zonulin levels in the blood and an increase in the permeability of the gut-blood barrier.[8] In recent studies, it has been observed that individuals with ADHD, ASD, bipolar disorder, and schizophrenia have higher serum levels of zonulin in comparison to control groups.[5,15,16] Claudins are the most important transmembrane proteins for TJ formation, function, and persistence.[17] There are 27 types of claudin, and claudin 5 is the most important claudin regarding the formation of the BBB.[18] Although claudin-5 is synthesized in many organs, it is most concentrated in the brain and lungs.[19] Claudin-5 seals the paracellular space through its interaction with other claudins in neighboring cells. Claudin-5 prevents the passage of small molecules from the paracellular space by ensuring almost no distance between the cells.[20] Studies have reported that the serum claudin-5 levels of subjects diagnosed with ADHD, obsessive–compulsive disorder, and bipolar disorder are higher than in control groups.[21,22,23] Meanwhile TJ are also susceptible to degradation induced by inflammatory mediators such as cytokines.[7] Interferon-gamma (IFN-γ) and interleukin-17A (IL-17A) are believed to impact the intestinal epithelial and BBB.[24,25] These cytokines have been linked to the development of nervous system diseases like multiple sclerosis, epilepsy, and ischemic brain injury, as well as neurodevelopmental disorders such as ASD and ADHD.[26,27,28]

Although the brain–intestinal axis has gained considerable attention in the causation of neurodevelopmental disorders, to our knowledge, there has been no study investigating zonulin, claudin-5, IFN-γ, and IL-17A serum levels in children with SLD. The current study seeks to address this gap by comparing the serum levels of zonulin, claudin-5, IFN-γ, and IL-17A in children with SLD and a control group. We hypothesized that (a) serum levels of zonulin, claudin-5, IFN-γ, and IL-17A will be higher in the study group compared to the control group and (b) serum levels of zonulin and claudin-5 will be higher in the study group regardless of levels of the inflammatory biomarkers (IFN-γ and IL-17A).

MAIN POINTS

- SLD is quite common neurodevelopmental disorders of childhood, its aetiology has yet to be fully explained.
- In recent years, studies on the relationship between intestinal and blood-brain brain barrier permeability and psychiatric disorders have increased.
- Serum zonulin and claudin-5 levels were higher in the SLD group compared to the control group.
- Serum IFN-γ and IL-17A levels did not differ between the groups.

MATERIAL AND METHODS

Participants and Procedures

Participants of this study were enrolled in the outpatient clinic of the Department of Child and Adolescent Psychiatry at the Istanbul Faculty of Medicine, Istanbul University. The study group included children, aged 6 to 11 years, who have been referred and followed up with a diagnosis of SLD according to DSM-5 criteria. A total of 63 children diagnosed with SLD were invited to participate in the study. Finally, 43 subjects with SLD were included in the study (11 children refused to give a blood sample and 9 children were excluded because of having a comorbid diagnosis of ADHD and/or ASD). The control group consisted of age- and gender-matched children referred to the outpatient pediatrics clinics at the Istanbul Faculty of Medicine for routine visits. Exclusion criteria for the study and control groups included the presence of major medical comorbidities (e.g., metabolic/genetic/autoimm une diseases, neurological disorders, gastrointestinal disorders, visual or hearing disability, etc.), schizophrenia, bipolar disorder, ADHD, ASD, and an intelligence quotient (IQ) score below 80 according to the Turkish version of the Wechsler Intelligence Scale for Children-Revised (WISC-R).[29] Children with a history of psychotropic medication, non-steroidal anti-inflammatory drugs, and proton pump inhibitor use within the previous 6 months and current or recent history of infectious diseases were also excluded.

Subjects who gave verbal assent and whose parents presented written informed consent participated in the study following a complete description of the study. In addition to clinical evaluations based on DSM-5, the SLD-clinical observation battery (SLD-COB) was used to confirm and exclude SLD diagnosis in the study and control groups, respectively.[30] The WISC-R was also performed with all participants during psychometric evaluation to exclude the presence of intellectual disabilities. The application and evaluation of these tests were performed by experienced psychologists with specific training and experience in their application. Child and adolescent psychiatrists in the research team evaluated all participants for comorbid psychiatric disorders using the Schedule for Affective Disorders and Schizophrenia for School-Age Children–Present and Lifetime Version–DSM-5 (K-SADS-PL-DSM-5). Sociodemographic data

forms developed by the researchers were completed by the parents of all participants. The study was approved by the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date and Number: 29/09/2020-165171), and all procedures were in accordance with the standards in the Declaration of Helsinki.

Measures

Special Learning Disorder-Clinical Observation Battery

The SLD-COB was initially developed by Korkmazlar in 1992 and then revised to involve new subscales. Nine subscales that assess reading/writing/arithmetic skills, Gessell figures, ability to draw a clock, right-left discrimination, lateralization, before-after relationships, and ordering are included. Each subscale is described as being consistent or inconsistent with SLD.[30] In addition to supporting DSM-5-based SLD diagnosis, we also used the data gathered from the SLD-COB to assess the presence and the severity of impairment in reading.

Wechsler Intelligence Scale for Children-Revised

This scale was established to determine the IQ levels of children between 6 and 16 years. Initially, it was developed by Wechsler as the Wechsler-Bellevue scale to assess adults. Then the WISC was arranged for children in 1949 based on this adult scale. The WISC-R is a version of the original WISC that was revised in 1974. The Turkish adaptation of the WISC-R was developed by Savaşır and Şahin.[29] This scale consists of verbal and performance parts, and IQ scores are identified in 3 parts: verbal, performance, and full-scale IQ.

Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version-DSM-5

Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime is a semi-structured diagnostic instrument developed by Kaufman et al (1997) according to DSM-IV criteria to assess the present and lifetime psychopathology in children and adolescents.[31] The Turkish adaptation of the updated version of K-SADS-PL according to DSM-5 criteria was conducted by Unal et al.[32] Test-retest reliability coefficients were between 0.63 and 0.82. Interrater reliability coefficients were between 0.63 and 1.0.[32]

Blood Sampling

Venous blood samples of the subjects were drawn between 8:00 and 10:00 AM to prevent circadian variation. Participants were also asked to abstain from heavy exercise, eating, and drinking 8 hours prior to sampling. Venous blood samples were collected in biochemical tubes. The serum was then obtained by centrifugation at 3000 rpm for 10 minutes. The serum specimens were stored at -80°C until analysis. Serum levels of zonulin, claudin-5, IFN-γ, and IL-17 were measured by the enzyme-linked immunosorbent assay (ELISA) method using commercial human ELISA test kits and following the protocols of the manufacturer (Elabscience Biotechnology Inc, TX, USA).

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences Statistics version 21.0 (IBM SPSS Corp.; Armonk, NY, USA). Data were demonstrated as counts, frequencies, median (25-75 percentiles), and mean ± standard deviation. Comparisons of categorical variables between groups were analyzed by using Pearson's chi-square test. The Shapiro-Wilk test was used to determine the normality of the continuous variables. For non-normally distributed continuous variables, the differences between groups were analyzed using the Mann-Whitney *U* test. When the data were normally distributed, the Student's *t*-test was performed. The multivariate analysis of covariance (MANCOVA) was used to compare biomarkers, which were normally distributed and the variance and covariance matrices were homogeneous, between the two groups controlling for age, sex, and body mass index (BMI). The correlation between continuous variables was examined by Spearman's rank correlation analysis. Probability values (*P*) smaller than .05 were considered statistically significant.

RESULTS

There were no significant differences in terms of age, gender, and BMI between the 2 groups. The sociodemographic and clinical characteristics of the subjects are shown in Table 1.

According to the SLD-COB in the study group, 59.5% (n=25) of the children had specific impairments in reading, writing, and arithmetic; 31% (n=13) had specific impairments in both reading and writing; and 9.5% (n=4) had specific impairments only in reading. About 41.9% (n=18) of the study group had comorbid psychiatric diagnoses which included specific phobias (n=10), social anxiety disorder

Table 1. The Sociodemographic and Clinical Characteristics of the Subjects

Variables	Study Group (n=43)	Control Group (n=43)	*t*/z/*x*2	*P*
Age (years (median (IQR))	10 (8-10)	9(8-10)	−1.13	.26[a]
Gender (Male n(%))	33 (76.7%)	33 (76.7%)	1.00	1.00[b]
Body mass index (mean ± SD)	17.5 ± 3.8	18.4 ± 3.3	2.33	.234[c]
WISC-R VIQ (mean ± SD)	89.28 ± 11.51	107.10 ± 10.15	7.30	<.001[c]
WISC-R PIQ (mean ± SD)	103.44 ± 14.04	111.35 ± 13.85	2.52	.014[c]
WISC-R FSIQ (median (IQR))	94 (88-103)	108 (102-116)	−5.65	<.001[a]

Non-normal continuous data are presented as median and as 25-75 percentiles.
FSIQ, full-scale intelligence quotient; PIQ, performance intelligence quotient; VIQ, verbal intelligence quotient; WISC-R, Wechsler Intelligence Scale for Children-Revised; IQR, interquartile range.
[a] Mann-Whitney *U* test, [b]chi-square test, [c]Student's *t*-test.

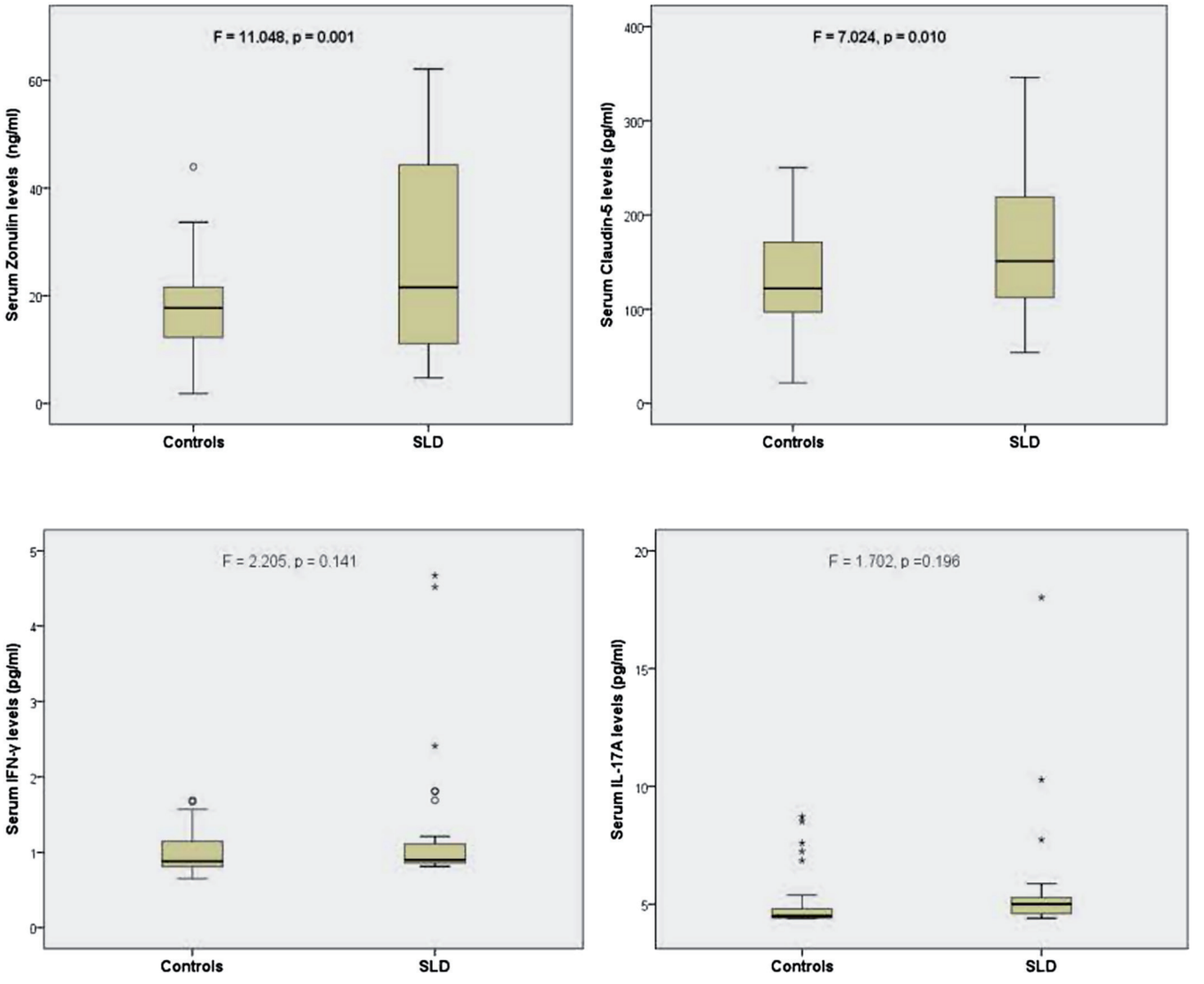

Figure 1. The distribution of serum levels of zonulin, claudin-5, interferon-gamma, and interleukin-17A in patients with specific learning disorder and controls.

(n = 5), enuresis (n = 5), communication disorders (n = 4), and separation anxiety disorder (n = 2).

Serum zonulin and claudin-5 levels of the study group were significantly higher than the control group according to the MANCOVA test while controlling for age, gender, and BMI. On the other hand, serum IFN-γ and IL-17A levels were not significantly different between the 2 groups (Figure 1 and Table 2). There was no correlation either between levels of zonulin and IFN-γ ($r = -.100$, $P = .525$) and IL-17A ($r = -.102$, $P = .516$) or claudin-5 and IFN-γ ($r = -.186$, $P = .234$) and IL-17A ($r = -.157$, $P = .314$).

Table 2. Serum Levels of Zonulin, Claudin-5, Interferon-Gamma, and Interleukin-17A

Variables	Study Group# (n = 43)	Control Group# (n = 43)	MANCOVA[a]		
			F	*P*	ηp^2
Zonulin (ng/mL)	27.4 ± 19.1	17.5 ± 9.1	11.1	**.001**	0.12
Claudin-5 (pg/mL)	167.2 ± 73.1	128.2 ± 57.2	7.02	**.010**	0.08
IFN-γ (pg/mL)	1.2 ± 0.8	1.0 ± 0.3	2.21	.141	0.03
IL-17A (pg/mL)	5.4 ± 2.2	5.0 ± 1.1	1.70	.196	0.02

Statistically significant values are given in bold.
IL-17A, Interleukin-17A; IFN-γ, Interferon-gamma; MANCOVA, multivariate analysis of covariance.
[a]Covariates: age, sex, body mass index; #data presented as mean ± SD.

DISCUSSION

In this study, we aimed to investigate serum levels of zonulin, claudin-5, IFN-γ, and IL-17A in children with SLD. To our knowledge, this is the first study investigating the levels of biomarkers that are linked with permeability of gut-blood and BBBs in SLD. We have found several important findings which may have further clinical and research implications. First, we found that serum zonulin

and claudin-5 levels are significantly higher in subjects with SLD compared to the control group. Second, there was no correlation between levels of zonulin and claudin-5 and those of inflammatory biomarkers such as IFN-γ and IL-17A.

Despite the gut–brain axis attracting much attention in the etiology of neurodevelopmental disorders during recent years, the lack of such studies in subjects with SLD may be an important gap. There have been a number of studies investigating serum levels of zonulin, a biomarker for intestinal permeability, and claudin-5, a biomarker for the BBB, in the etiology of neurodevelopmental and psychiatric disorders.[5,15,16,21,22,23]

As the primary regulator of the permeability of the gut-blood and also BBB, zonulin is used as a clinical indicator of gut permeability, and high zonulin serum levels are positively correlated with increased permeability of the gut–blood barrier.[8,14] In addition to several neuropsychiatric disorders (such as ASD, ADHD, bipolar, schizophrenia, obsessive-compulsive disorder), zonulin serum levels were also increased in obesity, obesity-related insulin resistance, and diabetes types 1 and 2.[5,33]

Recent studies have reported that serum zonulin levels were increased in subjects with ASD and the severity of ASD was correlated with zonulin levels.[34,35] However, another study reported significantly lower serum zonulin levels in the ASD group.[36] Similarly, some other studies reported higher serum zonulin levels in children with ADHD compared to control groups and a positive correlation between zonulin levels and ADHD symptom severity.[4] However another recent study reported no significant difference in serum zonulin levels in subjects with ADHD.[23] In addition, Usta et al[37] reported significantly higher serum zonulin levels in patients with schizophrenia, and Kılıç et al[22] reported significantly higher zonulin levels in patients with bipolar disorder than healthy controls. Regarding claudin-5, several studies have reported higher claudin-5 levels in subjects with ADHD, with bipolar disorder, and with obsessive-compulsive disorder;[21,22,23] however Usta et al[37] reported decreased claudin-5 levels in patients with schizophrenia compared to healthy controls. However, there has been no study, to the best of our knowledge, on serum levels of zonulin and claudin-5 in SLD.

In our study, consistent with our hypothesis, we found that both zonulin and claudin-5 levels were significantly higher in subjects with SLD. These findings may show that consistent with the previous reports on other neurodevelopmental and psychiatric disorders, gut–blood and BBB permeability may be impaired in SLD. Impaired gut–blood and BBB permeability may be associated with SLD symptoms in several ways. The gut microbiota may affect learning and memory functions.[38,39] Disruption of the intestinal barrier may cause intestinal microbiota and their metabolites to pass from the lumen into the circulatory system. Undesired particles in the circulatory system might trigger immune system cells to produce proinflammatory cytokines. These cytokines could interrupt cognitive functioning.[40] Immune activation is thought to be one of the mechanisms of action of the gut–brain axis.[38] Further studies are needed to explain the role of gut–blood and BBB permeability in the etiology of neurodevelopmental disorders.

Meanwhile, we found no significant differences in serum levels of inflammatory mediators (such as IFN-γ and IL-17A) which are thought to have effects on the intestinal epithelial and BBB.[24,25] However, a literature review revealed that there are only a few studies investigating IFN-γ and IL-17A serum levels in the etiology of neurodevelopmental disorders. Saghazadeh et al[27] reported that serum IFN-γ levels were higher in subjects diagnosed with ASD compared to the control group. A very recent study did not find significant differences in plasma levels of IFN-γ and IL-17A in children with ADHD.[27] In the present study, we did not detect any significant differences in serum levels of IFN-γ and IL-17A between the study and control groups. Additionally, consistent with our hypothesis, we found no correlation either between zonulin and IFN-γ and IL-17A or claudin-5 and IFN-γ and IL-17A levels. Therefore, the increase in zonulin and claudin-5 levels may not be due to inflammation, which is measured by IFN-γ and IL-17A but is a finding directly related to SLD. Zonulin and claudin-5 may be biomarkers showing impaired intestinal and BBB permeability in subjects with SLD. Further research is needed to elaborate on this finding in the etiology of neurodevelopmental disorders.

The current study has several strengths and limitations. This is the first study in the literature investigating serum zonulin and claudin-5 levels together with inflammatory cytokines such as IFN-γ and IL-17A. We tried to have a homogenous group by excluding possible confounding factors such as comorbid neurodevelopmental disorders, major medical comorbidities, and medication use. In addition, besides detailed psychometric assessment, all cases had a clinical examination by experienced child psychiatrists. The results of the present study should be evaluated with certain limitations in mind. The cross-sectional nature of the study limits the understanding of whether there is a cause–effect relationship. The relatively small sample size may be another limitation. Although zonulin and claudin-5 are frequently studied biomarkers of intestinal and BBB permeability, we did not use a direct method to evaluate intestinal and BBB permeability. We also could not exclude several psychosocial factors that may affect serum zonulin, claudin-5, IFN-γ, and IL-17A levels.

High serum zonulin and claudin-5 levels may show impaired gut–blood and BBB permeability in the etiology of SLD. This first report in the literature may have several clinical and research implications. Further research is needed to determine whether zonulin and claudin-5 may be biomarkers, and some dietary interventions or specific

agents such as zonulin or claudin-5 inhibitors could be used in the management of neurodevelopmental disorders including SLD.

Ethics Committee Approval: This study was approved by Clinical Research Ethics Committee of Istanbul University (Approval No: 165171, Date: September 29, 2020).

Informed Consent: Written informed consent was obtained from the parents of the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

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