INDICATORS OF IMPAIRED PERMEABILITY AND INFLAMMATION IN THE INTESTINE IN CHILDREN WITH AUTISM SPECTRUM DISORDER OF THE REPUBLIC OF UZBEKISTAN

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Abstract. *The association of dysbacteriosis with diseases of the central nervous systemhas been proven in the last 10 years [1]. Microbial changes cause an increase in intestinal permeability, and the penetration of bacterial fragments and toxins induces local and systemic inflammatory processes affecting distant organs, including the brain [3,4,6]. Thus, the integrity of the intestinal epithelial barrier plays a central role in the microbiota-gut-brain axis. In this review, we discuss recent evidence for faecal calprotectin, an important regulator of intestinal epithelial cell tight contacts, which is thought to play a key role in maintaining blood-brain barrier function [3,4,10].*

Keywords: *autism spectrum disorder, microbiota, calprotectin, zonulin,rehabilitation.*

Autism spectrum disorders (ASD) are a set of complex neurodevelopmental disorders defined by behavioural impairment in social interaction, delayed and impaired speech, repetitive or stereotyped behaviour and restricted interests [10,11,13]. ASDs represent a serious public health problem. The WHO estimates that globally, one child in 160 has an autism spectrum disorder [10,11,13]. Manyindividuals with ASD have symptoms of co-morbidities including seizures, sleep problems, metabolic disorders and gastrointestinal disorders that have significant implications for health, development, social development and education. Gastrointestinal complaints are a frequent concern for parents and can be associated with problem behaviours and other medical problems such as sleep dysregulation (ATN Annual Report, unpublished data, November 2009). Despite the extent of these problems, potential gastrointestinal problems are not usually considered in the assessment of ASD. This probably reflects several factors, including the variability of reported indicators [6,7].

In 2000, a research group from Fasano reported the discovery of zonulin, a human protein analogue of Zonula occludens (Zot)-derived cholera vibrio (Zot) toxin, which regulates paracellular permeability through protein kinase C (PKC)- dependent rearrangement of actin microfilaments and impairment of ZO-1 structure [5,10]. Since then, the influence of the zonulin pathway on the regulation of intestinal permeability has been confirmed by phase 2 clinical trials demonstrating the beneficial effects of the zonulin antagonist larazotide acetate (AT-1001) in patients with celiac disease [2]. Although most of our knowledge about zonulin is related to intestinal diseases, its importance in almost all our organs including brain, heart, lung, kidney, liver, skin, etc. has been described so far. [11,12,13,14]. Indeed, dysbiosis is associated with increased release of zonulin in the gut, impaired intestinal permeability, and activation of inflammatory mediators. The spread of microbial fragments, toxins, and inflammatory factors, including zonulin, originating from the gut eventually reaches distant organs, including the central nervous system, resulting in increased blood-brain barrier (BBB) permeability, neuroinflammation

and behavioural changes that partially go away. by depletion of the microbiota [13,14]. Together, these suggest that dysbiosisand the zonulin pathway may be central factors in MGBA-related diseases. Thus faecal calprotectin is a cytoplasmic protein present mainly in neutrophils; it is released as a result of cell death and destruction [5]. In some inflammatory processes, calprotectin is released with intracellular exudate in large amounts and can be detected in body fluids and serum; hence, it can be considered as a useful marker of inflammation [6]. Calprotectin in faeces indicates infiltration of the intestinal tract by neutrophils. Faecal calprotectin (FC) levels correlate with intestinal tract inflammation histologically and macroscopically [4,5]. FC is considered a non-invasive marker of some gastrointestinal disorders that can be used before more invasive procedures [7]. Some studies have shown that gutinflammation is more prevalent in children with autism, while other studies have failed to detect gut inflammation in children with autism.

Objective: The aim of the work was to study faecal calprotectin and zonulin levels as a markerof inflammation and intestinal permeability in children with autism and its possible association with gastrointestinal manifestations and their relationship withsymptom severity.

Methods: 38 children with ASD aged 2 to 8 years who met the DSM-5 diagnostic criteria [8] were included in the study. A control group of 38 healthy children matched for sex and age was also included to compare their faecal calprotectin and zonulin levels with the case group. Cases were selected among those who received outpatient rehabilitation treatment at the Republican Children's Psychoneurological Hospital named after U.K. Kurbanov.The children were referred to the Prime Medical Centre to submit biologicalmaterial. Written informed consent was obtained from the children's parents/guardians after explaining the stages and nature of the study. Patients with dysmorphic features suggestive of syndromal developmental delay and children with any chronic gastrointestinal disease such as chronic gastritis or celiac disease were excluded. All examined children were subjected to a thorough history taking and a complete physical examination with special emphasis on neurological examination. The severity of autism was assessed using the Child Autism Rating Scale (CARS and MCHAR-T) [9]. It was categorised as mild or moderate ifbetween 30 and 36.5, or severe if greater than or equal to 37.

Gastrointestinal (GI) symptoms and symptom severity were assessed using a modified version of the GI Severity Index, that is, a shortened version called the 6- GI Severity Index (6-GSI) [10]. It included 6 items: constipation, abdominal pain, diarrhoea, stool odour, stool consistency and flatulence. Each option received a score of 0, 1, or 2 depending on its frequency per week; a score of zero for any option was interpreted as no symptom, and a score of 1 or 2 for any option indicated the presence of a symptom of varying severity. A total score equal to or less than 3 was categorised as a low score and more than 3 as a high score.

We used ELISA (ELISA-immunoassay) method, which was designed for the quantification of calprotectin and zonulin. The assay used a two-way sandwich method with two selected monoclonal antibodies that bind to human calprotectin. Standards, control samples and pre-diluted patient samples being assayed for human calprotectin were added to microplate cells with a high homogeneousmonoclonal antibody to human calprotectin and zonulin applied to the microplate. In the first incubation step, calprotectin is bound by the immobilised antibody. Peroxidase-labelled conjugate is then added to each cell and the following combination was formed: immobilised antibody - human calprotectin - peroxidase- labelled conjugate. Tetramethylbenzidine (TMB) was used as peroxidase substrate.Finally, acidic stop reagent was added to complete the reaction. The colour was changed from blue to yellow. The brightness of

the yellow colour was directly proportional to the concentration of calprotectin in the sample. We created a dose- response curve of the optical unit (optical density - OD at 450 nm) versus concentration using values obtained from the standard. Calprotectin present in patient samples was determined directly from this curve.

Faecal samples were collected and stored at temperatures below -20°C. The conformity assessment procedure follows Annex 2 of Directive 98/79/EC on in- vitro diagnostic medical devices. Certification according to EN ISO 13485:2012+ AC 2012-ISO 13485 2003 +ISO 13485:2016(E) Coll.1: Medical devices- Quality management system. FC levels were categorised as follows: <50 μg/g = normal, ≥50 μg/g = elevated.

Comparisons were made between cases and controls regarding faecal calprotectin and zonulin levels, and the following correlations were investigated among cases: between autism severity (CARS) and gastrointestinal symptom severity (6-GSI), FC and gastrointestinal symptom severity (6-GSI), and between FC and autism severity (CARS).

Statistical Analyses: Statistical calculations of the obtained data were carried out using parametric and non-parametric analysis methods. Microsoft Office Excel 2016 spreadsheets were used to collect correction, editing, classification, and visual presentation of the obtained results. Free computing environment software R (v.3.5.1) was used to perform statistical analysis of the data. The Shapiro-Wilk criterion (number of subjects <50) or the Kolmogorov-Smirnov criterion (number of subjects >50), as well as the asymmetry and kurtosis indices were used to compare the obtained quantitative values (parameters, indicators) to determine whether these values conform to the normal distribution law.

The reliability of the results was assessedat the 5% level. The χ 2 test was used to test the relationship between qualitative variables. Fisher's exact correction or Monte Carlo correction was used when more than 20% of cells in the χ 2 had an expected number less than 5 and required correction. The Mann-Whitney criterion was used to compare two investigated independent subgroups that were not normally distributed.

Results Obtained: Out of 38 children with ASD, 28 (70.0%) were male and 12 (30.0%) were female. Their ages ranged from 2 to 8 years.

Cases were diagnosed between 14.0 and 36.0 months of age with a mean of 23.40 ± 5.07 months. Twenty-six (65%) children hadregressive type of autism and 14 (35%) had non-regressive autism. According to CARS and MCHAR-T, 23 (57.5%) children with ASD had mild to moderate and 17 (42.5%) had severe ASD. CARS ranged from 30 to 48.5 with a mean of 36.18 ±5.22.

Gastrointestinal symptoms were present in 33 (82.5%) children with ASD. Stool odour and constipation were the most frequent symptoms (70%), while diarrhoea was the least frequent (17.5%). The total score on 6-GSI was low in 26 (65%) cases and high in 14 (35%) cases. The total score ranged from 0 to 9 with a meanof 2.85 ± 2.05.

A control group of 38 healthy children matched for sex and age was included to compare their faecal calprotectin levels with the patient group. Faecal calprotectin levels were elevated (≥ 50 μg/g) in 14 (35%) children with ASD and 10 (25%) of the control group.

Comparing the mean faecal calprotectin levels, it was shownthat the mean FC level of cases was 47.03 ± 26.68 whereas in the control group it was 37.08 ± 21.55 and this showed a statistically significant difference (p = 0.049)

Table 1

Fecal calprotectin	**ASD (38)**	**Control (38)**	**Test sigma**	**P-value**
	H %	H %		
< 50	25 66,9	38 76,8	x 2 = 0,991	0,401
≥ 50	13 35	11 26		
Min Max.	13,0-100,0	11.35-90.0	U = 595,5*	0,049*
Mean ± standard deviation	47,03 ±26,68	37,08 ±21,55		
Median (IQR)	36,0	27.20		

χ 2 - chi-square test, U - Mann-Whitney test, p value for comparison between study groups, *statistically significant at p ≤ 0.05.

A control group of 40 healthy children matched for sex and age was included to compare their faecal zonulin levels with the patient group. Faecal zonulin levels were elevated (≥120 μg/g) in 33 (65%) children with ASD and 12 (27%) of the control group. Comparing the mean faecal zonulin levels, it was shown that the mean level of FZ in cases was 120 ± 99.68 whereas in control group it was 82.08 ± 61.55 and this showed a statistically significant difference (p = 0.049)

Table 2

Fecal zonulin	**ASD (38)**	**Control (38)**	**Test sigma**	**P-value**
	H %	H %		
< 83	120 97,9	85 84,8	x 2 = 1,899	0,501
≥ 50	33 36	12 27		
Min Max.	12,0-105,0	12.41-80.0	U = 494,5*	0,058*
Mean ± standard deviation	85,03 ±66,68	82,08 ±61,55		
Median (IQR)	76,0	6.20		

χ 2 - chi-square test, U - Mann-Whitney test, p value for comparison between study groups, *statistically significant at p ≤ 0.05.

Discussion: Gastrointestinal problems are a common pathology in children with ASD; numerous studies have suggested a probable gut-brain connection that can be explained by inflammatory, immunological, or genetic factors [2]. The gut-brain afferent pathway involves inflammatory mediators, the entero-endocrine system, the gut microbiota, and sensory epithelial

cells, whereas the efferent pathway involves the neuroendocrine and autonomic nervous systems [13].

In the present study, we investigated faecal calprotectin and zonulin as a marker of inflammation and gastrointestinal permeability in children with RAS. It was found that faecal calprotectin and zonulin levels were elevated in 32 compared to 5 controls. This finding is in agreement with the findings of Karkelis et al. [14], de Magistris et al. [15], Babinska et al. [16] and Eduardo et al. [17] who observed that higher levels of calprotectin were found in the faeces of childrenwith autism than in normal children. Karkelis et al. conducted their study on 45 autistic children aged 2.5 to 8 years and found increased levels of faecal calprotectin in children with ASD compared to healthy children.

Regarding gastrointestinal manifestations, it was found that 82.5% of patients with ASD had at least one gastrointestinal symptom, with stool odour and constipation being the most common (in 70% of patients) and diarrhoea the least common (in 17.5% of patients). High 6-GSI values were observed in 35% of children with ASD.

Conclusion: Gastrointestinal manifestations are a frequent comorbidity in patients with autism, and the severity of their gastrointestinal manifestations' correlates closely with the severity of autism. Patients with ASD have significantly higher levels of FC and FZ than healthy controls, and their FC and FZ levels correlate closely with the severity of gastrointestinal manifestations in children with autism. FC as a laboratory marker, and the gastrointestinal severity score can be used as an indicator of the severity of gastrointestinal problems in autistic patients with gastrointestinal symptoms.

REFERENCES

1. Hoilat, G.J.; Altowairqi, A.K.; Ayas, M.F.; Alhaddab, N.T.; Alnujaidi, R.A.; Alharbi, H.A.; Alyahyawi, N.; Kamal, A.; Alhabeeb, H.; Albazee, E.; et al. Larazotide acetate for treatment of celiac disease: A systematic review and meta-analysis of randomized controlled trials. Clin. Res. Hepatol. Gastroenterol. 2022, 46, 101782. [Google Scholar] [CrossRef].
2. Smecuol, E.; Sugai, E.; Niveloni, S.; Vázquez, H.; Pedreira, S.; Mazure, R.; Moreno, M.L.; Label, M.; Mauriño, E.; Fasano, A. Permeability, zonulin production, and enteropathy in dermatitis herpetiformis. Clin. Gastroenterol. Hepatol. 2005, 3, 335–341. [Google Scholar] [CrossRef] [PubMed].
3. Uzzau, S.; Lu, R.; Wang, W.; Fiore, C.; Fasano, A. Purification and preliminary characterization of the zonula occludens toxin receptor from human (CaCo2) and murine (IEC6) intestinal cell lines. FEMS Microbiol. Lett. 2001, 194, 1–5. [Google Scholar] [CrossRef].
4. Molloy CA, Manning-Courtney P. Prevalence of chronic gastrointestinal symptoms in children with autism and autistic spectrum disorders. Autism 2003; 7: 165-71. [PubMed] [Google Scholar].
5. Kang V, Wagner GC, Ming X. Gastrointestinal dysfunction in children with autism spectrum disorders. Autism Res 2014; 7: 501-6. [PubMed] [Google Scholar].
6. Sandler RH, Finegold SM, Bolte ER, Buchanan CP, Maxwell AP, Väisänen ML, et al. Short-term benefit from oral vancomycin treatment of regressive-onset autism. J Child Neurol. 2000 Jul;15(7):429–35.

7. Nithianantharajah J, Balasuriya GK, Franks AE, Hill-Yardin EL. Using Animal Models to Study the Role of the Gut-Brain Axis in Autism. Curr Dev Disord Rep. 2017;4(2):28–36.
8. Szymanska, E.; Wierzbicka, A.; Dadalski, M.; Kierkus, J. Fecal Zonulin as a Noninvasive Biomarker of Intestinal Permeability in Pediatric Patients with Inflammatory Bowel Diseases—Correlation with Disease Activity and Fecal Calprotectin. J. Clin. Med. 2021, 10, 3905.
9. Stilling RM, Dinan TG, Cryan JF. Microbial genes, brain & behaviour – epigenetic regulation of the gut–brain axis. Genes, Brain and Behavior 2014; 13:69-86; PMID:24286462; http://dx.doi.org/ 10.1111/gbb.12109 [PubMed] [CrossRef] [Google Scholar]
10. Wang Y, Kasper LH. The role of microbiome in central nervous system disorders. Brain Behav. Immunology 2013; pii: S0889-1591(13)00600-4; PMID:24370461; http://dx.doi.org/ 10.1016/j.bbi.2013.12.015 [PMC free article] [PubMed] [CrossRef] [Google Scholar].
11. Dietert RR, Dietert JM, DeWitt JC. Environmental risk factors for autism. Emerg Health Threats J 2011; 4:7111; PMID:24149029; http//dx.doi.org/ 10.3402/ehtj.v4i0.7111 [PMC free article] [PubMed] [CrossRef] [Google Scholar]
12. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI, Cristofori F, Guerzoni ME, Gobbetti M, Francavilla R. Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. PLoS One 2013; 8:e76993; PMID:24130822; http://dx.doi.org/ 10.1371/journal.pone.0076993 [PMC free article] [PubMed] [CrossRef] [Google Scholar]
13. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan;505(7484):559–63.
14. Niehus R, Lord C. Early medical history of children with autism spectrum disorders. J Dev Behav Pediatr. 2006 Apr;27(2 Suppl):S120–7.