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Research Article

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Assessment of lipocalin-1, resistin, cathepsin-D, neurokinin A, agmatine, NGF, and BDNF serum levels in children with Autism Spectrum Disorder

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Abstract

Objectives: Autism Spectrum Disorder (ASD) is a neurological and developmental disorder that affects all aspects of social communication, with stereotypical and limited interest and atypical responses to sensory stimuli. There isn't a reliable lab test that can help clinicians diagnose ASD. This study aimed to examine serum lipocalin-1, resistin, cathepsin-D, neurokinin A, agmatinase, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial fibrillary acidic protein (GFAP) levels in children with ASD.
Methods: The study had 40 matched control subjects and 39 ASD sufferers in total. The diagnosis was made by medical professionals using the Gilliam Autistic Disorder Rating Scale-2-TV (GOBDÖ-2-TV), Denver Developmental Screening Test, and Porteus Intelligence Tests.
Results: Compared to the control group, serum levels of lipocalin-1, resistin, cathepsin-D, agmatinase, BDNF, and NGF were found to be high and GFAP levels were low in children having ASD. B12 and magnesium levels were significantly lower in the ASD group than in the control group, while sodium and chloride levels were higher in the ASD group (p<0.05).
Conclusions: The aforementioned parameters may be involved in the pathophysiology of ASD, as evidenced by the fact that their values were greater in children with ASD than in controls. Additionally, evaluation of some routinely measured biochemical parameters may suggest that nutritional and electrolyte balance may differ in individuals with ASD.

Keywords: Autism Spectrum Disorder; BDNF; ELISA; GFAP; resistin

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Introduction

Repetitive habits, limited interests, and social communication deficits are characteristics of Autism Spectrum Disorder (ASD), a complex neurological illness. ASD, which begins in early childhood, is a lifelong disease [1]. The World Health Organization (WHO) forecasts that ASD affects one in 160 children globally on average, with males being affected at a rate four to five folds higher than females [1, 2]. ASD's precise cause is yet unknown. Nonetheless, it has been acknowledged that a complex interplay and combination of immunological dysfunction, genetics, and environmental variables may contribute to the development of ASD [3]. A member of the neurotrophic family, which also contains neurotrophic factors 3 and 4 and nerve growth factor (NGF), is brain-derived neurotrophic factor (BDNF) [4]. Since BDNF's primary roles include regulating glycogenesis, neurogenesis, and synaptogenesis as well as short- and long-term synaptic interactions that impact neuroprotection, memory processes, and cognition, it has received special attention in the study of ASD [5]. Numerous studies demonstrate that autistic children have higher blood, serum, and brain BDNF levels than typical controls

[6]. The characteristic intermediate filament protein of astrocytes and an indicator of astroglial activity is glial fibrillary acidic protein (GFAP). The cerebrospinal fluid of children with autism, Rett syndrome, and several neurological disorders has been shown to have high quantities of this protein [7]. Moreover, recent studies have supported the promising role of GFAP level in blood as a diagnostic and prognostic biomarker of brain and spinal cord disorders [8, 9].

This study aims to examine the levels of important biochemical parameters such as lipocalin-1, resistin, cathepsin-D, neurokinin A, agmatinase, BDNF, NGF and GFAP in children with ASD. The second aim of the study is to evaluate whether some of these parameters can be used as biomarkers in the diagnosis of ASD.

Materials and methods

Participants

Before the study, it was determined that the minimum required sample size should be a minimum of 40 people in the groups, having a power of 80 % and a type 1 error of 0.05, based on the study of Barbosa et al. [10, 11]. The sample of the study occurs 39 children between the ages of 2–10 applied to the Şırnak State Hospital Child and Adolescent Psychiatry outpatient clinic between June 1, 2023 and March 29, 2024, and were diagnosed with ASD according to DSM-V. The control group consists of 40 healthy children between the ages of 2–10 who applied to Şırnak State Hospital Pediatrics clinic between June 1, 2023, and March 29, 2024. Individuals were not included in the sample if the families of the patients did not volunteer for the study, did not sign informed consent, or if the parent who volunteered for the study had a diagnosis of physical/mental retardation, psychotic disorder, bipolar disorder or ASD that would prevent them from understanding and answering the questions asked during the clinical interview and filling out the scale. Additionally, individuals with a diagnosis of severe immunological disease were not included in the sample. Before taking part in the research, the patient's first-degree relatives or legal guardians gave their consent by the Declaration of Helsinki decisions. The Siirt University Non-invasive Clinical Research Ethics Committee granted the required permits before the study. (Date: 28.04.2023 No: 2023/04/01/03).

Clinical evaluation

Psychiatric examination of all patients included in the study was performed by child psychiatrists. Diagnostic evaluation was made according to DSM-V (Handbook of Differential Diagnoses). Individuals diagnosed with autism were evaluated for autism severity using the Gilliam Autistic Disorder Rating Scale-2-TV (GOBDÖ-2-TV). For the developmental and mental evaluations of the patients, the Denver Developmental Screening Test was applied in the 0–6 age group, and the Porteus Intelligence Test was applied by the child development specialists of our clinic for the mental evaluations of individuals over the age of 6. Mental and developmental assessments were clinically reevaluated by child psychiatrists. Information about the children was obtained from the participants' families using a sociodemographic data form.

Socio-demographic data form

This form consists of questions about children's age, gender, physical/mental diseases, developmental characteristics, and contact information.

Gilliam Autistic Disorder Rating Scale-2-TV (GOBDÖ-2-TV)

Originally known as the Gilliam Autism Rating Scale-2, it was created in the United States by James Gilliam and underwent revisions and editing in 2006 [12, 13]. The Turkish validity and reliability of the scale was evaluated by Diken et al. [14]. GOBDÖ-2-TV is a Likert-type scale scored with a four-point scale, aiming to evaluate children between the ages of 3–23 who exhibit behaviors that characterize autistic disorder. Scale; it consists of 42 items collected under three subscales: Stereotypic Behaviors (14 items), Communication (14 items), and Social Interaction (14 items). Categorically, autistic disorder index evaluation data of GOBDÖ-2-TV results; 69 and below: Not likely to be seen. 70–84: There is a possibility of being seen. 85–99: Mild autism. 100–114: Moderate autism. 115 and above: It is considered as severe autism [14].

Denver II developmental screening test

Denver II Developmental Screening Test (DIIGTT) is frequently utilized around the world to provide information about the general development of the child by evaluating the age-appropriate skills of healthy children between the ages of 0–6. It is based on 12 methods of direct evaluation. It is divided into four sections with 134 items to assess language, fine motor skills, gross motor abilities, and personal-social skills. The test's Turkish standardization of was made by Anlar, Yalaz [15].

Porteus labyrinths test

It is a performance-based intelligence test developed by Porteus [16] to determine the individual's planning and adaptation skills to innovations. The test requires the ability to use paper and pencil, and the test has no time limit and no literacy requirement [17]. Turkish validity-reliability study was conducted by Toğrol [18]. The test used to evaluate children, teenagers and adults consists of 12 mazes.

Sample collection

Following a 10 to 12-h overnight fast, the subjects' fasting venous blood samples were obtained between 8 and 10 a.m. Blood samples were collected from the brachial veins into yellow cap serum tubes with gel (Vacusera, 5 mL, 235305; Disera A.S, Izmir, TR). The samples were centrifuged at 2,683 g for 10 min at 4 °C. The biochemical (c8000, Abbott, Abbott Park, IL, USA) tests were performed in the Medical Biochemistry Laboratory of Şırnak State Hospital. The collected serum samples were split into parts, put in an eppendorf tube, and labeled in order to determine the serum concentrations of additional molecules. Until they were examined, the samples were kept at −80 °C.

Measurement of serum levels of molecules

Agmatinase (agmatine ureohydrolase), BDNF, cathepsin-D, GFAP, lipocalin-1, neurokinin A, NGF and resistin levels were determined by using human ELISA kits (Wuhan Feiyue Biotechnology Co., Ltd., Wuhan, Hubei, China). The Limit of quantification values respectively of the tests according to the content of the kits are 2.5 pg/mL, 9.38 pg/mL, 7.5 pg/mL, 15 pg/mL, 0.3 ng/mL, 1 μg/mL, 0.5 pg/mL and 1 ng/mL. Analysis was performed by Elisa Plate Reader (Thermo Scientific, Multiskan GO, SN: 1510–02723, USA) according to the manufacturer's instruction. Inter- and intra-assay coefficient variances (%CV) of all kits were <10 %. This study's ELISA analyses were all carried out in the Siirt University Science and Technology Application and Research Center lab. Serum B12, ferritin and folate levels were analyzed using the electrochemiluminescence method with a roche cobas device and an immunoassay system. Calcium was measured with the Arsenazo III method, magnesium with the enzymatic method, and sodium, chloride and potassium were measured with the Abbott ARCHITECT c8000 biochemistry autoanalyzer using the ion-selective electrode (ISE) method.

Statistical analysis

Statistical analysis was performed using the SPSS 25.0 package program (SPSS, Version 21.0. Armonk, NY: IBM USA). To determine if the data in the groups were normally distributed, the Shapiro-Wilk and Kolmogorov-Smirnov tests were employed. The Mann-Whitney U test was applied to nonparametric data in order to compare mean serum levels between groups. The correlations between the data were examined using Spearman's correlation analysis. Descriptive statistics, median, minimum and maximum data are presented for numerical variables. A value of $p<0.05$ was thought to be significant.

Results

Of the patients participating in the study, 74.4% were boys and 25.6% were girls. Boys made up 47.5 % and girls made up 52.5 % of the control group. Table 1 shows the comparative biochemical results of the patients and the control groups. Compared to the control group, the patient group exhibited elevated levels of sodium and chloride, whereas magnesium and vitamin B12 levels were reduced. At the $p<0.05$ level, median comparisons of these values are statistically significant.

Serum levels of lipocalin-1, resistin, cathepsin-D, agmatinase, BDNF, and NGF were high in the patients, and this difference was statistically significant when comparing the median values of serum levels of biomolecules between the patient and control groups in this study. The patient group's GFAP serum level was found to be statistically substantially decreased (Table 2) ($p<0.05$).

According to the results of the correlation analysis performed in the control group, strong positive relationships were found between lipocalin-1 and resistin, cathepsin D, agmantinase, GFAP and NGF. The correlations of lipocalin-1 with resistin ($r=0.550$, $p<0.001$), cathepsin D ($r=0.671$, $p<0.001$), agmantinase ($r=0.422$, $p=0.007$), GFAP ($r=0.483$, $p=0.002$) and NGF ($r=0.415$, $p=0.008$) were found to be statistically significant. In the correlation analysis of the patient group, a significant positive relationship was found between agmantinase and lipocalin-1 ($r=0.386$, $p=0.015$). In addition, resistin; correlations with cathepsin D ($r=0.555$, $p<0.001$), neurokinin A ($r=0.420$, $p=0.008$), GFAP ($r=0.643$, $p<0.001$) and NGF ($r=0.582$, $p<0.001$) were also found to be statistically significant.

Table 1: Distribution of age and some routine biochemistry parameters.

Variables	Control (n=40)			ASD (n=39)			p-Value
	Median	Min	Max	Median	Min	Max	
Age, years	6	2	10	8	3	10	0.397
B12, pg/mL	395	222	878	310	119	862	0.025
Ferritin, ng/mL	36	5	175	30	2	110	0.173
Folate, ng/mL	12	5	19	10	1	23	0.441
Calcium, mg/dL	10	8.9	10.3	9.6	8.71	10.5	0.443
Magnesium, mg/dL	2.11	1.85	2.35	2.07	1.78	2.41	0.027
Sodium, mmol/L	139	133	142	140	134	143	0.018
Chloride, mmol/L	106	101	109	107	104	111	0.030
Potassium, mmol/L	4.35	0.57	5.30	4.27	3.51	6.17	0.937

Comparison was made using the Mann-Whitney U test; Min, minimum; Max, maximum; ASD, Autism Spectrum Disorder.

Table 2: Serum levels of molecules compared between groups.

Variables	Control (n=40)			ASD (n=39)			p-Value
	Median	Min	Max	Median	Min	Max	
Lipocalin1, ng/mL	8.79	6.07	16.46	13.09	8.90	18.32	**0.001**
Resistin, ng/mL	20.6	13.50	51.45	29.08	10.04	60.86	**0.008**
Cathepsin-D, pg/mL	248.24	152.34	652.93	311.44	183.65	572.30	**0.006**
Neurokinin A, μg/mL	16.12	7.32	28.40	16.93	8.61	35.07	0.533
Agmatinase, pg/mL	137.33	99.03	249.18	220.50	112.74	485.57	**0.001**
BDNF, pg/mL	472.58	23.50	4,627.83	1,327.25	42.28	4,968.03	**0.003**
GFAP, pg/mL	979.81	425.74	1,952.04	770.93	395	2,325.19	**0.012**
NGF, pg/mL	6.87	3.84	37.15	10.20	3.74	25.60	**0.022**

Comparison was made using the Mann-Whitney U test; Min, minimum; Max, maximum; ASD, Autism Spectrum Disorder; BDNF, brain derived neurotrophic factor; GFAP, glial fibrillary acidic protein; NGF, nerve growth factor.

When the disease severity levels of ASD patients participating in the study were evaluated, it was found that most of the patients were at the moderate level. According to clinical data, 74.4 % of ASD patients were mentally retarded. 56.4 % of the patients showed signs of self-mutilation, while 66.7 % of the patients showed signs of coherent sentence formation.

Discussion

The purpose of this study was to characterize the concentrations of certain biomarkers in the serum of both healthy control subjects and those with ASD, as well as to investigate the potential of biomarkers to aid in the diagnosis of ASD. The results of this study showed that serum lipocalin-1, resistin, cathepsin-D, neurokinin A, agmatinase, BDNF, NGF, and GFAP levels were statistically different in healthy controls and children diagnosed with ASD.

Lipocalin-1 has been defined as a molecule that can bind various lipophilic molecules and eliminate harmful lipids and lipophilic molecules from the body [19]. Because of our hypothesis that lipocalin-1 may play a part in immunological processes in autistic individuals, In this investigation, we discovered that the lipocalin-1 levels of the ASD group were statistically substantially greater than those of the control group. Our results are in line with a research published in the literature that found lipocalin-1 abnormalities in saliva samples from autistic people, which may be connected to immune dysregulation processes in autism [20]. Our findings are consistent with a research that measured lipocalin-1 using tear samples from patients with Alzheimer's disease and another that measured lipocalin-1 using tear samples from patients with diabetic retinopathy, one of the neuroinflammatory illnesses. These studies revealed that the patient group had higher lipocalin-1 levels than the control group, suggesting that tears could be used as a biomarker [21, 22].

Resistin is a proinflammatory cytokine that plays a role in the pathogenesis of various inflammatory central nervous system disorders. Resistin, secreted from adipose tissues, monocytes, and macrophages, is important due to its roles in metabolic functions and immunoinflammatory system [23, 24]. In our study, resistin levels in the ASD group were found to be statistically significantly higher than those in the control group (p=0.008). The results of this study are in line with earlier research that suggests pro-inflammatory cytokine resistin expression may rise *in vitro*, as well as a study that was published in the literature and suggests that immunological and mitochondrial variables may be involved in the genesis of autism [25, 26]. Another study has shown increases in resistin and visfatin and levels in ASD patients compared to the control group. Therefore, our results indicate that immune dysfunctions may be associated with ASDs [27].

Neurogenic inflammation is mediated by numerous neuropeptides, primarily tachykinins. It has been established that the neuropeptides known as neurokinin A, tachykinins – substance P and neurokinin B are released from the excitatory section of non-adrenergic, non-cholinergic excitatory nervous system nerves in response to allergen exposure [28]. Mammalian neuronal tissue contains the decapeptide neurokinin A, which has the following sequence: His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH2. [29]. Numerous studies have revealed that, in response to inflammatory stimuli, non-neuronal cells, including immune cells, can also produce tachykinins. By changing several facets of immune cell activity, these tachykinins significantly affect inflammatory responses [30]. According to a study, during asthma exacerbations, children's blood and sputum eosinophil counts significantly correlated with elevated sputum neurokinin A levels [28]. Therefore, the rise in serum neurokinin A levels in autistic children may be due to immune cell activation after exposure to certain environmental antigens (such infectious agents, dietary allergies, and heavy metals) and the ensuing increase in release. The results of this study corroborate previous research by showing that the neurokinin A levels were higher in the autism spectrum group when compared to the control group.

Cathepsin D, the main lysosomal aspartic acid protease, is extensively expressed in the brain and is quite selective in hydrolyzing certain peptide bonds of target proteins [31]. There have been suggestions of multiple functions for cathepsin D both inside and outside of the cell, as well as for its proteolytically inactive precursor, pro cathepsin D. Chronically inflamed organs and malignant tumors have elevated levels of procathepsin D. It has been demonstrated that cathepsin D triggers apoptosis through caspase-8 and is crucial for controlling cellular apoptosis [31–33]. Furthermore, recent studies have shown that cathepsin D may be released into the cytosol upon inducing apoptosis and cleave the Bcl2 family member Bid, which releases cyt c from mitochondria and activates caspase-9 and -3 [34]. Additionally, a number of studies have shown that procathepsin D causes the production of inflammatory cytokines, including IL-4, IL-8, IL-10, and IL-13 [35]. However, it has been shown that inflammatory cytokines such as TNF-α and IFN-γ increase extracellular pro cathepsin D in primary endothelial cell cultures [36]. In our current findings, it was determined that the cathepsin D level in children diagnosed with autism spectrum was higher than in the control group. Based on these, it could be hypothesized that high cytokine levels may cause increased cathepsin D expression in the autistic brain and that cathepsin D may mediate cytokine-induced apoptosis.

Agmatine is a naturally occurring polyamine metabolite that is produced by arginine decarboxylase from the amino acid L-arginine. When agmatine is metabolized by the agmatinase enzyme, it is converted into putrescine, spermidine and spermine [37]. Agmatine has been shown in numerous prior studies to have neuromodulatory, anti-apoptotic, neurogenic, antioxidant, and anti-inflammatory activities [38, 39]. Also, agmatine has demonstrated beneficial effects on many preclinical studies assessing its therapeutic effects, including hypoxic ischemia, epileptic seizures, morphine tolerance, anxiety disorders and memory, depression, and neuropsychiatric conditions like Parkinson's illness [40]. Numerous animal experiments have demonstrated that treating mice with agmatine reduces the symptoms of obsessive-compulsive disorder [41, 42]. In an experimental animal study, it was suggested that the increase in agmatinase caused by the upregulation of the agmatinase gene and the resulting agmatine deficit led to a depressive phenotype [43]. According to another study, it was determined that agmatinase expression increased significantly in postmortem measurements in individuals with bipolar and unipolar disorders, and it was stated that agmatine degradation may be responsible for the pathogenesis [44]. According to the results of the current study, children with ASD had greater levels of agmatinase than the control group.

BDNF belongs to the neurotrophic family, which also includes NGF and neurotrophic factors 3 and 4 [45]. BDNF has drawn particular focus in the research of ASD because to its significant functions in regulating glycogenesis, neurogenesis, and synaptogenesis as well as short- and long-term synaptic contacts that affect neuroprotection, memory functions, and cognition [46]. Serum levels of BDNF were found to be greater in children with ASD than in the control group. A statistically significant positive change was observed. In this study, there was a noteworthy positive association between the patients' blood levels of BDNF and agmatinase. Another study found that the average BDNF levels in the sick group were considerably greater than those in the control group, which supports our findings [47]. According to the findings of a meta-analysis research that measured five neurotrophic factors in 2,486 children with ASD and 4,303 healthy controls, elevated peripheral blood concentrations of BDNF, NGF, and VEGF were identified as an indication of ASD in children. This result confirms clinical data showing children with ASD have aberrant neurotrophic factor profiles [48]. According to a different study, autistic children had higher levels of BDNF in their brain, blood, and serum when compared to normal controls [49].

The activation of astrocytes is indicated by GFAP, the distinctive intermediate filament protein of astrocytes. It has been discovered that children with Rett syndrome, autism, and other neurological disorders have elevated amounts of this protein in their cerebral fluid [50]. Furthermore, recent research has confirmed the blood level of GFAP as a promising biomarker for brain and spinal cord disorders, both in terms of diagnosis and prognosis [51, 52]. In a study, it was found that autistic people's brains had noticeably greater amounts of GFAP. This data suggests that autism is associated with microglial and astrocyte activation. and is associated with astrocyte activation [53]. The buildup of GFAP molecules in the intracellular space and decreased microglial and astrocyte activation may be indicated by low blood GFAP levels in children with autism. Our study's comparison of the GFAP levels of the ASD and control groups revealed that the ASD group's GFAP levels were statistically substantially lower than the control group's. Nerve growth factor is a multifunctional molecule that influences energy homeostasis, synaptic plasticity, and neuro-immune processes. It is currently unclear how NGF-induced neuronal differentiation impacts ASD, despite the fact that its processes are widely characterized [54]. NGF levels were examined in a 2013 research including 49 children with ASD and 49 healthy children; the findings indicated that the ASD group's NGF levels were noticeably greater than the control group's [55]. It was consistent with our results in another study that plasma NGF levels of the ASD group were significantly higher than those of the control group [56].

Conclusions and recommendations

This study investigated the relationships between agmatinase, BDNF, neurokinin A, cathepsin D, lipocalin-1, resistin, GFAP, and NGF levels, as well as some essential routine biochemical parameters of ASD patients. While several studies have looked into biomarkers in the etiology of ASD, none have evaluated all eight of these biomarkers together. This is significant because this study is the first in the literature. Understanding the pathophysiology of autism can be greatly aided by identifying molecular alterations in the disorder through experimental research.

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