ЦИТОКИНЫ СЛЮНЫ ПРОТИВ ПЛАЗМЫ КАК ВОЗМОЖНЫЕ ПРЕДИКТОРЫ ТЯЖЕСТИ АУТИЗМА У ДЕТЕЙ

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Резюме. Расстройства аутистического спектра (РАС) являются широко распространенными, гетерогенными нарушениями нейроразвития с множественной этиологией, подтипами и траекториями развития, для которых отсутствуют доступные и эффективные биомаркеры. Важным фактором риска, способствующим дефициту развития нервной системы, наблюдаемому при РАС, является иммунная дисфункция, которая проявляется в том числе дисбалансом цитокинов в мозге и на периферии. В последние годы в качестве биологического материала для диагностики РАС предложена слюна, что обусловлено доступностью и неинвазивностью метода ее получения. Вместе с тем, вопрос о том, могут ли уровни цитокинов в слюне быть использованы в качестве эффективных ранних биомаркеров аутизма требует дополнительных исследований, в том числе сравнений: слюна против плазмы/сыворотки крови.

Цель – провести сравнительный анализ уровней цитокинов: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, в слюне и плазме крови для выделения возможных маркеров РАС и их тяжести у детей.

В исследование было включено 11 детей с типичным нейроразвитием (ТРД) и 55 детей с диагнозом РАС, среди которых 37 человек имели легкую или умеренную степень тяжести аутизма (по CARS), а 18 детей – тяжелую. У всех детей одномоментно были собраны образцы не стимулированной смешанной слюны и венозной крови. Концентрации цитокинов: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, слюне определяли multiplex Luminex™ analysis. Плазменные уровни тех же цитокинов оценивали с помощью ELISA. Различия между группами проверяли с помощью U-критерия Краскела–Уоллиса, с апостериорными попарными сравнениями по Коноверу–Инману, между образцами (плазма/слюна) – парного критерия Уилкоксона. Наличие зависимости между концентрациями цитокинов в плазме и слюне определяли с помощью линейной регрессии методом RMA.

Во всех обследуемых группах уровни IL-6, IFNγ и IL-10 в слюне были ниже, а TNFα, IL-1β и IL-4 – выше, чем соответствующие уровни тех же цитокинов в плазме. Не зависимо от состояния здоровье/болезнь, значимых корреляций между уровнями цитокинов в слюне и плазме у детей не обнаружено. Значимых различий в концентрациях цитокинов слюны между детьми с легкой и тяжелой степенью РАС не обнаружено, однако уровни IL-1β были значимо ниже, а IL-10 – выше, в слюне обеих групп детей с РАС, по сравнению с ТРД.

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Таким образом, цитокины слюны могут быть использованы в качестве маркеров РАС у детей, но не тяжести состояния. Отсутствие корреляций в уровнях некоторых про-/противовоспалительных цитокинов между слюной и плазмой крови, вероятно, может свидетельствовать об особом иммунологическом статусе экологической ниши – ротовая полость.

Ключевые слова: цитокины, слюна, плазма, расстройства аутистического спектра, дети, диагностика

SALIVA VERSUS PLASMA CYTOKINES AS POSSIBLE PREDICTORS OF AUTISM SEVERITY

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Abstract. The autism spectrum disorders (ASD) are now widely accepted as a pervasive, complex, heterogeneous neurodevelopmental disorders with multiple etiologies, subtypes, and developmental trajectories. There are no available and effective biomarkers for them. Immune dysfunction is seen as an important risk factor contributing to the neurodevelopmental deficit in ASD, and is signified, among other things, by an imbalance of cytokines in the brain and on the periphery. In recent years, saliva has been proposed as a biological material for diagnosing ASD, due to the accessibility and non-invasiveness of the method for its production. However, the question of whether salivary cytokine levels may be used as effective early biomarkers for autism requires further research, including saliva versus plasma/serum comparisons.

Aim: a comparative analysis of the levels of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, in saliva and blood plasma to identify possible markers of ASD and their severity in children.

The study included 11 children with typical neurodevelopment (TDC) and 55 children with ASD, among whom 37 children had mild or moderate autism (according to CARS), and 18 children had severe autism. Samples of unstimulated mixed saliva and venous blood were simultaneously collected from all children. Salivary concentrations of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10 were determined by multiplex Luminex™ analysis. Plasma levels of cytokines were assessed by ELISA. Differences between groups were tested using the Kruskal–Wallis U-test with post-hoc Conover–Inman comparisons, between samples (saliva/plasma) are using the Wilcoxon signed-rank test. The correlation between the concentrations of cytokines in plasma and saliva was determined using linear regression by the RMA method.

In all examined groups, the levels of IL-6, IFNγ and IL-10 in saliva were significantly lower, and TNFα, IL-1β and IL-4 were higher than the corresponding levels of the same cytokines in plasma. Regardless of health/disease status, no significant correlations were found between salivary and plasma cytokine levels in children. IL-1β levels were significantly lower and IL-10 levels were higher in the saliva of both groups of children with ASD compared with TDC. No significant differences in salivary cytokine concentrations were found between children with mild and severe ASD.

Thus, salivary cytokines can be used as markers of ASD in children, but not the severity of the condition. The absence of correlations in the levels of some pro/anti-inflammatory cytokines between saliva and blood plasma may probably indicate a special immunological status of an ecological niche, the oral cavity.

Keywords: cytokines, saliva, blood plasma, autism spectrum disorders, children, diagnosis

Introduction

The autism spectrum disorders (ASD) is now widely accepted as a pervasive, complex, heterogeneous neurodevelopmental disorders with multiple etiologies, subtypes, and developmental trajectories [8]. Currently there are no available diagnostic biomarkers and the diagnosis of ASD is based on typical features that include repetitive behaviors, and impaired social communication and interaction [11]. The etiology of ASD is largely unknown but, in most cases, likely due to a combination of genetic and environmental factors [1]. Compounding evidence supports the role of maternal immune system activation (MIA), particularly due to infection, at the specific periods of gestation as a risk factor for autism [10]. The exact molecular pathways that lead from MIA to ASD are not clear, however, a fair amount of research indicates that cytokines and chemokines may be the key elements in this process [13]. Normally, cytokines regulate growth, cell proliferation and synaptogenesis in nervous tissue. Cytokines also modulate host responses

to infection, injury, inflammation and diseases of uncertain etiology [7]. However, the results of the studies addressing serum or plasma levels of cytokines in autism appear to be inconsistent, probably due to these inconsistencies reflect the heterogeneity of the ASD diagnosis [12].

In addition, the invasiveness and painfulness of the procedure combined with the neuropsychological features of children with ASD provides difficulties in obtaining plasma samples. In recent years, a new direction has been developing – the use of saliva as a biological material for the diagnosis of a number of pathological conditions, including autism [3, 5], which is due to the accessibility of biological material and the non-invasive method of obtaining it.

According to the current protocol for assessing the immunity of saliva, it is a biological fluid with a high potential of antibacterial and antiviral molecules. Saliva contains a large number of immune cells, among which 95% are heterogeneous neutrophils, and the rest are lymphocytes and myeloid cells. The antigen-presenting cells, neutrophils in the network of cells of the innate immune system, T- and B-lymphocytes are predominant in the oral mucosa. Moreover, saliva plays an important role in the regulation of the oral microbiome. Salivary mucin, antimicrobial peptides and proteins, produced by salivary neutrophils, help innate selection of bacterial colonization and biofilm formation [4]. However, the question of whether salivary cytokine levels may be used as effective early biomarkers of neurodevelopmental diseases requires further research, including saliva versus plasma/serum comparisons.

Aim: a comparative analysis of the levels of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, in saliva and plasma to identify possible markers of ASD and their severity in children.

Materials and methods

A total of 66 children participated in the study. The study comprised 55 children diagnosed with autism spectrum disorders (ASD, male / female ratio 43/13, age range 3-13 years) and 11 neurotypical children (TDC) (male/ female ratio 9/2, age range 4-13 years). The ASD children met the International Classification of Diseases 10th Revision (ICD-10) criteria for Childhood autism (F84.0) and Atypical autism (F84.1), which were determined a child psychiatrist and a psychologist. Using the Childhood Autism Rating Scale (CARS), children with ASD were divided into 2 groups: 37 people with mild or moderate autism (mean CARS score 32.0±1.5) and 18 people with severe autism (mean CARS score 39.0±3.4). The protocol for this study was approved by the Bioethics Committee of Chelyabinsk State University (2/2019). Written informed consent was obtained from the parents of each child before any study procedure was carried out.

Unstimulated mixed saliva was collected from all children between 8:00 and 9:00 a. m., by passive flow in SaliCaps tubes (IBL International, Germany). One hour before sampling, ASD and TDC children refrained from eating, drinking, mouth rinsing, and teeth brushing. Venous blood samples in a volume of 4 mL were collected at the same time in vacuette tubes with K_3EDTA. Blood was centrifuged for 10 min at 3000 rpm to obtain plasma. Saliva and plasma samples were stored frozen at -70 °C until assayed.

The concentrations of the cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10 in saliva were determined by multiplex Luminex™ analysis, using a 41-plex Human Cytokine/Chemokine Magnetic Bead Panel (Merck, Millipore) and a MAGPIX analyzer (Luminex). Plasma levels of the cytokines were measured using the commercial ELISA kit (JSC Vector-Best, Novosibirsk, Russia) on a MultiscanEX plate analyzer.

The statistical analysis was carried out with PAST software (v. 4.03). Data are presented as medians and interquartile ranges (IQR). The differences between groups were checked using the Kruskal–Wallis test with post-hoc Conover–Inman comparisons, between samples (plasma/saliva) were using the Wilcoxon signed-rank test. The correlation between the concentrations of cytokines in plasma and saliva was determined using linear regression by the RMA method.

Results and discussion

The results of the assessment of the concentration of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10 in the saliva and plasma of children with ASD and TDC are summarized in the Table 1.

We found that in all the examined groups of children (regardless of the state of health/disease), the levels of IL-6, IFNγ and IL-10 in saliva were lower, and the levels of TNFα, IL-1β and IL-4 were higher than the corresponding levels of the same cytokines in plasma. All differences were statistically significant except for IL-6 concentrations in the TDC group (Table 1). This may indicate the determination of the immune response by the humoral type in saliva and by the cellular type in plasma.

Analysis of the levels of some pro/anti-inflammatory cytokines in saliva depending on the presence/absence of ASD and its severity showed that the concentrations of IL-1β were significantly lower, and the concentrations of IL-10 were significantly higher in the saliva of children with ASD, compared with TDC (Table 1). Thus, in the saliva of children with ASD, we showed the activation of the anti-inflammatory potential, whose indicators may be used as markers of autism, but not of the severity of the condition.

In plasma, in contrast to saliva, children with severe ASD have reduced concentrations of early

TABLE 1. CYTOKINE LEVELS IN PLASMA AND SALIVA OF TDC AND CHILDREN WITH MILD / SEVERE ASD, Me ($Q_{0.25}$-$Q_{0.75}$)

Parameters (pg /mL) / source	TDC	mild ASD	severe ASD
IL-6 / saliva	0.89 (0.67-1.57)	1.28 (0.92-1.69)	1.45 (0.71-2.57)
IL-6 / plasma	2.29 (0.89-3.00) $W_{(11)}$ = 54.0; p = 0.060	1.89 (0.67-3.06) $W_{(37)}$ = 507.0; p = 0.019	3.83* ** (2.31-5.78) $W_{(18)}$ = 146.0; p ≤ 0.001
IFNγ / saliva	0.49 (0.28-0.80)	0.83 (0.59-1.44)	0.59 (0.44-1.36)
IFNγ / plasma	11.13 (8.91-11.15) $W_{(11)}$ = 66.0; p = 0.003	10.48 (9.00-11.48) $W_{(37)}$ = 703.0; p = 0.001	14.58* ** (11.99-15.93) $W_{(18)}$ = 171.0; p ≤ 0.001
TNFα / saliva	6.58 (4.10-7.83)	7.27 (3.37-8.99)	7.0 (3.59-12.94)
TNFα / plasma	2.24 (1.53-3.40) $W_{(11)}$ = 66.0; p = 0.030	2.69 (2.36-3.60) $W_{(37)}$ = 637.0; p = 0.001	1.29* ** (1.17-1.69) $W_{(18)}$ = 165.0; p ≤ 0.001
IL-1β / saliva	60.85 (43.43-101.64)	5.60* (2.04-14.06)	8.18* (3.77-46.55)
IL-1β / plasma	3.07 (1.59-3.55) $W_{(11)}$ = 65.0; p = 0.004	3.20 (2.54-4.32) $W_{(37)}$ = 532.0; p = 0.006	1.86* ** (1.62-2.09) $W_{(18)}$ = 165.0; p ≤ 0.001
IL-4 / saliva	10.89 (6.67-31.36)	9.46 (5.95-13.67)	10.89 (6.67-30.01)
IL-4 / plasma	2.22 (1.80-2.35) $W_{(11)}$ = 66.0; p = 0.003	2.72* (2.40-3.07) $W_{(37)}$ = 690.0; p = 0.001	2.21** (2.00-2.46) $W_{(18)}$ = 170.0; p ≤ 0.001
IL-10 / saliva	1.15 (0.69-1.50)	2.33* (1.61-3.57)	2.94* (1.20-6.54)
IL-10 / plasma	9.68 (6.47-11.84) $W_{(11)}$ = 66.0; p = 0.004	7.75 (6.38-10.42) $W_{(37)}$ = 636.0; p = 0.001	5.91* ** (3.20-8.50) $W_{(18)}$ = 136.0; p = 0.028

Note. ASD, autism spectrum disorders; TDC, typically developing children. *, statistically significant differences between the indicators of the groups "TDC" and "mild ASD" / "severe ASD" (p ≤ 0.05); **, statistically significant differences between the indicators of the groups "mild ASD" and "severe ASD" (p ≤ 0.05). W is the value of the Wilcoxon test, p is the level of significance of differences between the cytokine values in plasma and saliva.

inflammatory response cytokines – TNFα, IL-1β and anti-inflammatory – IL-10, against the background of an increased concentration of late inflammatory response cytokines – IL-6 and IFNγ, possibly supporting systemic low-grade inflammation (Table 1). Thus, plasma cytokines are relevant predictors of ASD severity in children.

An assessment of the links between the concentrations of cytokines in saliva and plasma showed no significant correlations, regardless of the presence/absence of ASD and its severity (Figure 1).

Different concentrations of cytokines and the lack of correlations between salivary and plasma parameters suggest that salivary cytokine levels are not representative of plasma concentrations in children, regardless of the presence/absence of neurodevelopmental disorders. Although 30% of salivary proteins are filtered out of plasma [6], cytokines are too large to enter saliva via either diffusion or ultra-filtration. Therefore, these molecules enter saliva via leaky patches, such as tissue damage sites and inflammation, as well as crevicular fluid. Maintenance of the basic level of cytokines in saliva occurs with the help of the immune components of the mucous membranes of the oral cavity [2].

It is known that the oral cavity is the entrance gate to the human body. Commensal microorganisms, airborne antigens/allergens, and foodstuffs initially enter the oral mucosa before entering the gastrointestinal tract and respiratory tract. For the normal functioning of the body, the local immune system of the oral cavity provides a delicate balance: it provides effective immune surveillance without an excessive inflammatory response, and at the same time tolerance to commensals and harmless antigens [9]. Violation of this balance towards inflammation can lead to serious consequences for the body as a whole. Therefore, the anti-inflammatory potential of saliva in children with ASD and the absence of correlations in the levels of some pro/anti-inflammatory cytokines between saliva

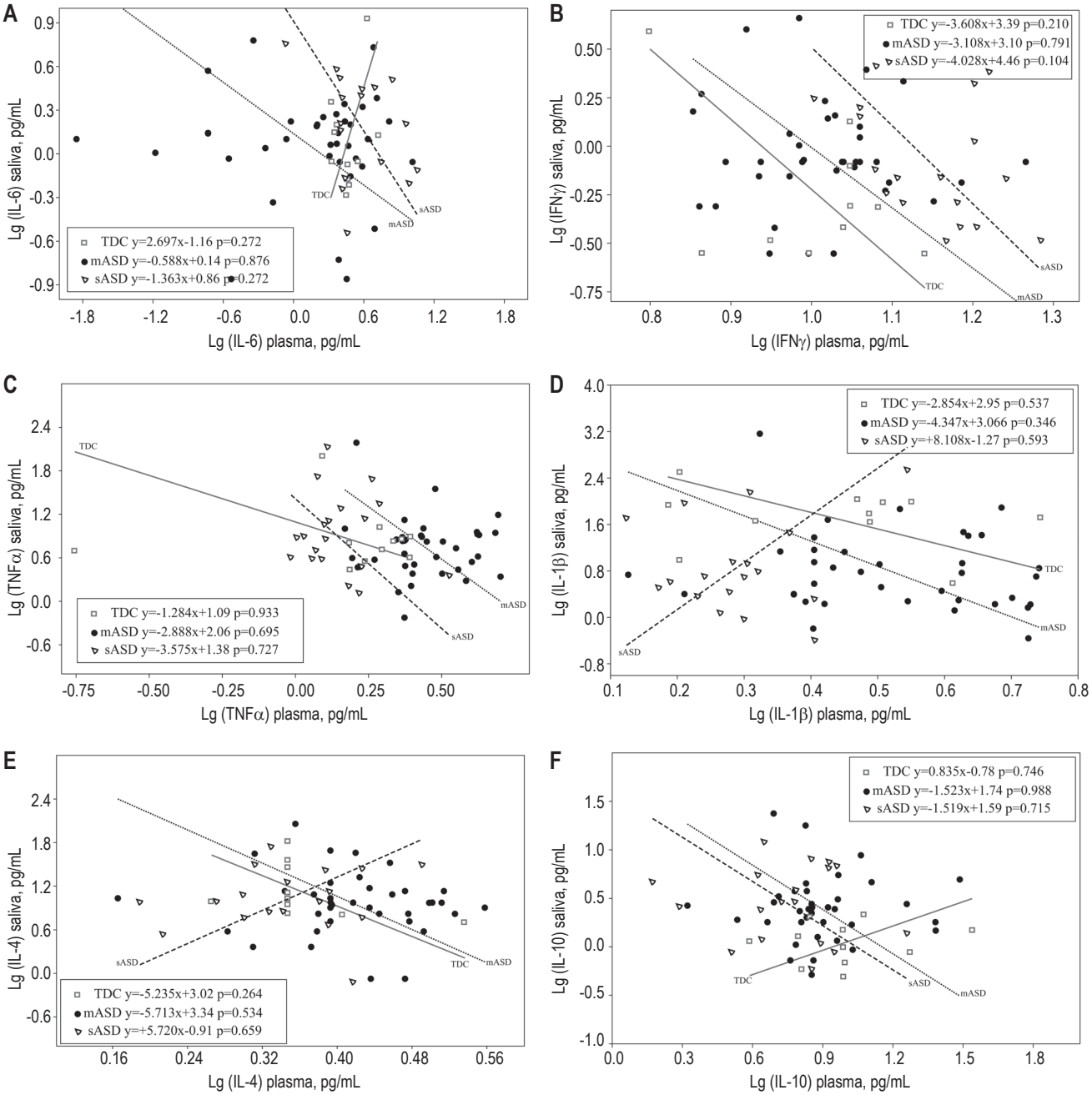

Figure 1. Linear regression between salivary and plasmatic levels of cytokines in typically developing children (TDC) and children with mild/severe autism (mASD/sASD)

Note. Linear regression saliva versus plasma cytokines: (A) IL-6, (B) IFNγ, (C) TNFα, (D) IL-1β, (E) IL-4, (F) IL-10 are shown. TDC, typically developing children; mASD, childen with mild autism; sASD, childen with severe autism Cytokine concentrations are converted to base logarithm 10. The lines show the regression equations for the TRD – solid line, midl ASD – dots line, severy ASD – long dashes line. Values cytokines for TDC are shown by squares, mild ASD by dots, severe ASD by triangles. y = ax+b – regression equation. p, the level of significance of the corelations between saliva and plasma.

and blood plasma, shown in our pilot study, can probably indicate the effectiveness of the regulatory functions of the ecological niche, the oral cavity.

Conclusion

Thus, saliva and plasma are two completely different systems, with their own development programs, structural components and functions. Changes of salivary cytokine concentrations do not correlate with plasma cytokine levels and do not reflect ASD severity in children. It is probably necessary to talk about the study of salivary immunome, which reflects the homeostasis/destabilization of a unique local niche, its tissue immunity, which, like any tissue immunity, is a component of the axis of the immune-neuroendocrine systems and the microbiome of the digestive tract.

References

1. Ashwood P. Preliminary findings of elevated inflammatory plasma cytokines in children with autism who have co-morbid gastrointestinal symptoms. *Biomedicines, 2023, Vol. 11, no. 2, 436.* doi: 10.3390/biomedicines11020436.
2. Beigpoor A., McKinlay B.J., Kurgan N., Plyley M.J., O'Leary D., Falk B., Klentrou P. Cytokine concentrations in saliva vs. plasma at rest and in response to intense exercise in adolescent athletes. *Ann. Hum. Biol., 2021, Vol. 48, no. 5, pp. 389-392.*
3. Farah R., Haraty H., Salame Z., Fares Y., Ojcius D.M., Said Sadier N. Salivary biomarkers for the diagnosis and monitoring of neurological diseases. *Biomed J., 2018, Vol. 41, no. 2, pp. 63-87.*
4. Freire M., Nelson K.E., Edlund A. The oral host-microbial interactome: an ecological chronometer of health? *Trends Microbiol., 2021, Vol. 29, no. 6, pp. 551-561.*
5. Janšáková K., Kyselicová K., Ostatníková D., Repiská G. Potential of salivary biomarkers in autism research: a systematic review. *Int. J. Mol. Sci., 2021, Vol. 22, no. 19, 10873.* doi: 10.3390/ijms221910873.
6. Loo J.A., Yan W., Ramachandran P., Wong D.T. Comparative human salivary and plasma proteomes. *J. Dent. Res., 2010, Vol. 89, no. 10, pp. 1016-1023.*
7. Manzardo A.M., Henkhaus R., Dhillon S., Butler M.G. Plasma cytokine levels in children with autistic disorder and unrelated siblings. *Int. J. Dev. Neurosci., 2012, Vol. 30, no. 2, pp. 121-127.*
8. Masi A., DeMayo M.M., Glozier N., Guastella A.J. An overview of autism spectrum disorder, heterogeneity and treatment options. *Neurosci. Bull., 2017, Vol. 33, no. 2, pp. 183-193.*
9. Moutsopoulos N.M., Konkel J.E. Tissue-specific immunity at the oral mucosal barrier. *Trends Immunol., 2018, Vol. 39, no. 4, pp. 276-287.*
10. Nardone S., Elliott E. The Interaction between the immune system and epigenetics in the etiology of autism spectrum disorders. *Front. Neurosci., 2016, Vol. 12, no. 10, 329.* doi: 10.3389/fnins.2016.00329.
11. Sauer A.K., Stanton J.E., Hans S., Grabrucker A.M. Autism spectrum disorders: etiology and pathology. Ed. Grabrucker A.M., Exon Publications, 2021, Chapter 1. Available at: https://www.ncbi.nlm.nih.gov/books/NBK573613/.
12. Suzuki K., Matsuzaki H., Iwata K., Kameno Y., Shimmura C., Kawai S., Yoshihara Y., Wakuda T., Takebayashi K., Takagai S., Matsumoto K., Tsuchiya K.J., Iwata Y., Nakamura K., Tsujii M., Sugiyama T., Mori N. Plasma cytokine profiles in subjects with high-functioning autism spectrum disorders. *PLoS One, 2011, Vol. 6, no. 5, e20470.* doi: 10.1371/journal.pone.0020470.
13. Zawadzka A., Cieślik M., Adamczyk A. The role of maternal immune activation in the pathogenesis of autism: a review of the evidence, proposed mechanisms and implications for treatment. *Int. J. Mol. Sci., 2021, Vol. 22, no. 21, 11516.* doi: 10.3390/ijms222111516.

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