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Original article

Serum levels of macrophage migration inhibitory factor in children and adolescents with autistic disorders

Background: *There is growing awareness of an immunological involvement in children with autistic disorder (AD). Studies suggest that innate rather than adaptive neuroimmune responses are associated with autism. Macrophage migration inhibitory factor (MIF), being an upstream regulator of innate and adaptive immunity, could play a role in this disorder.*

Objective: *We aimed to study serum levels of MIF in a subset of children with autism and its relation to disease severity and important clinical manifestations of the disease.*

Methods: *The study included 21 children and adolescents diagnosed with AD with a mean age of 6.9± 2.9 years. Patients were neurologically evaluated and categorized into those with mild to moderate autism and those with severe disorder. In addition to assessment of cognitive abilities and electroencephalogram performance, MIF levels were measured in the sera of included patients and were compared to those of a matched control group.*

Results: *Levels of MIF were not significantly different in the patients and the control group. However, serum MIF was significantly reduced in patients with severe AD (z=2.197, P=0.029) compared to those with milder disease. Furthermore, there was a significant negative correlation between MIF levels and the degree of severity of the non-verbal communicative skills (r= -0.49, P=0.042). MIF levels were not different in patients with mental retardation, or abnormal electroencephalogram when compared to the rest of the patients.*

Conclusion: *Our study suggests the presence of immune dysfunction in the form of derangement in serum MIF levels in children with AD. Its levels were specifically decreased in a subset of patients with severe disorder compared to those with mild to moderate disease. Decreased serum levels of MIF in patients with AD seem to be associated with worsening of the non-verbal communicative skills which is one of the disturbed behavioral parameters of AD. Further research is warranted to study the precise relationship of immune derangement and both the etiopathogenesis and the behavioral components of AD and its therapeutic implications.*

Keywords: Autism; innate immunity; immune dysfunction; macrophage migration inhibitory factor**.**

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INTRODUCTION

In the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), autism is classified as one of the five pervasive developmental disorders (PDDs) and is characterized by impairments in the three domains of social interaction, communication, and repetitive behaviors.[1-2]

Autism or autistic disorder (AD) is thought to involve a complex interaction between multiple and variable susceptibility genes,[3] epigenetic effects,[4] and environmental factors.[5] Many believe that autism results when a genetically susceptible child is exposed to an environmental trigger. Research into the pathophysiology of autism suggests multiple potential mechanisms, further supporting the likelihood of different groups of autism.[6]

There is emerging evidence and growing concern that a dysregulated or abnormal immune response may be involved in some forms of AD. In general, the links between the immune and neurological systems are becoming increasingly well known. Aberrant immune activity during critical periods of brain and neuronal development could potentially play a role in neural dysfunction,

typical of autism. Several lines of research have shown abnormalities in the nature, extent, and regulation of the immune response in autism, including a skewed generation of antibodies, cytokines, and immune cells.[7-12]

For the survival of all living creatures, an appropriate and balanced immune response to invading micro-organisms is essential. Macrophage migration inhibitory factor (MIF) is a distinctive cytokine that is secreted by both the anterior pituitary and immune cells in response to surgical stress, injury, and sepsis.[13-14] Accumulating data clearly demonstrate the role of MIF in modulating the innate immunity.[15-16] MIF increases lipopolysaccahride (LPS) responsiveness by increasing toll-like receptor 4 (TLR4) levels, through a pathway that involves the transcription factor PU.1.[15] An additional mechanism for MIF's role in sustaining monocyte/ macrophage activation and proinflammatory cytokine production in innate immune responses has been provided by Mitchell et al.,[16] who observed enhanced apoptosis of activated macrophages in MIF knock-out (MIF -/-) mice. In a clinical setting, it is likely that MIF regulates both early host responsiveness (via TLR4) and late phase toxicity to LPS (via enhanced macrophage activation and proinflammatory cytokine production).[14]

Furthermore, there is some evidence demonstrating a role of MIF in regulating the adaptive arm of the immune response, as well. MIF stimulates Th1 immune activity and induces proinflammatory cytokines and amplification of macrophage function. Within the context of inflammatory responses, the Th1 pathway controls macrophage activity, which is the main source of proinflammatory cytokines. These proinflammatory cytokines are highly pleiotropic and stimulate neutrophil and macrophage function and, in addition, induce production of acute phase proteins.[17] The principle Th1 cytokine, namely IFN-γ, is a potent stimulator of monocyte chemoattractant protein-1, which further stimulates macrophage function.[17-18, 13]

Given the role it plays in both innate and adaptive immunity, and its widespread secretion, we were stimulated to investigate serum levels of MIF in a group of children and adolescents with autism in relation to disease severity, important disease manifestations and associated findings such as mental retardation and EEG abnormalities.

METHODS

Study population

This case control study was conduced on 21 children and adolescents with autistic disorder (AD) recruited from Pediatric Neurology and Pediatric Outpatient clinics of Ain Shams University in the period from the beginning of December 2007 to the end of March 2009. They were 16 males and 5 females. Their ages ranged between 3 and 14 years (mean± SD: 6.9± 2.9years).

Inclusion criteria:

Patients included in the study were fulfilling the criteria for the diagnosis of autism according to the DSM IV diagnostic criteria for research.[1] Patients with classic-onset and those with regressive autism were both included.

Exclusion criteria:

- Patients with associated neurological or metabolic disorders (e.g. cerebral palsy, tuberous sclerosis, ..etc), other than mental retardation.
- Patients with other chronic illness, e.g. automimmune diseases.
- Patients with intercurrent infection were excluded from the study till resolution of infection. At the time of venipuncture, all the study subjects were afebrile, were not on antibiotics, and had no evidence of acute microbial illnesses by physical examination.

Autistic patients were studied in comparison to 24 age- and sex- matched clinically healthy children and adolescents serving as healthy controls. Their mean age was 6.08± 3.01 years. They were the apparently healthy sibs of the children attending the Outpatients Pediatric Clinic, Faculty of Medicine, Ain Shams University for minor illness. None of the included control subjects had history or clinical findings suggesting a neuropsychiatric disorder, or other chronic disease. The study was approved by the local ethical committer. Consents were obtained from the legal guardians of the included subjects after explaining the nature of the study to them.

Clinical evaluation of patients with autism.

This was based on medical history taking from caregivers, clinical examination, and neuro-psychiatric assessment. In addition, disease severity was assessed using Childhood Autism Rating Scale (CARS),[19] which rates the child on a scale from 1 to 4 in each of 15 areas (relating to people; emotional response; imitation; body use; object use; listening response; fear or nervousness; verbal

communication; nonverbal communication; activity level; level and consistency of intellectual response; adaptation to change; visual response; taste, smell, and touch response and general impressions). According to the scale, children who have scored 30-36 have mild to moderate autism, whereas those with scores above 36 have a severe degree of autism.

Patients were evaluated for history suggestive of frequent infections (more than 6/year documented by a physician)[11]. Family history of autoimmune diseases was ascertained in controls and autistic subjects in an identical manner. Parents were asked if a first- or second-degree relative had received a diagnosis of specified autoimmune disorder. A list of autoimmune diseases with descriptions was provided.

Assessment of cognitive abilities of autistic children.

This was done by using Stanford-Binet test[20] to calculate the intelligence quotient. Subnormal intellectual function is diagnosed when intelligence quotient is below 70.

Performance of electroencephalogram for autistic children.

Sleep-deprived interictal electroencephalograms were performed for the included patients, whenever possible with photic and hyperventilation provocation.

Assessment of serum MIF levels for the patients and the control group

This assay uses the quantitative sandwich-type enzyme immunoassay technique (R&D Systems, Inc. Minneapolis, MN 55413, USA). The samples were run in a blinded manner, in parallel on the same run with the same internal standards. A monoclonal antibody specific for MIF has been precoated onto a microplate. Standards and samples were plotted into the wells and any MIF present is bound by the immobilized antibody. After washing away any unbound substance, an enzyme- linked polyclonal antibody specific for MIF was added to the wells. Following wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of MIF in the initial step. The color developed was stopped by adding an acidic stop solution. The intensity of the color produced in the procedure was measured in a microtiter plate reader at a wavelength of 450 nm.

Statistical Analysis

The results were analyzed by commercially available software package (Statview, Abacus concepts, Inc., Berkley, California). The data were nonparametric, thus, they were presented as median and interquartile range [between 25th and 75th percentiles]. Mann-Whitney test was used for comparison between these data. The $\chi 2$ test was used for comparison between qualitative variables of the studied groups. Spearman's rank correlation coefficient "r" was used to determine the relationship between different variables. For all tests, a probability (P) of less than 0.05 was considered significant.

RESULTS

Clinical data of the patients group

Clinical data of the included children and adolescents are summarized in Table (1). According to the childhood autism rating scale (CARS), 11 (52.4%) patients were classified as having mild to moderate autism and ten had severe autism. Autistic regression was identified in four (19%) of the included subjects, who lost previously acquired language and behavioral skills after 18-24 months of age.

Of the studied autistic children, 12 (57.1%) had subnormal intellectual function (intelligence quotient below 70); ten of them had mild mental retardation (intelligence quotient between □50 and 69). Seizures were reported in only four of the included autistic children (19%), all of them had severe autistic disorder. Clinically, the seizures pattern was focal in two patients, focal with secondary generalization in one and generalized-onset seizures in the fourth patient. Abnormal epileptiform encephalogram was detected in seven (33.3%) of the studied group (3 had clinical seizures). Most of the abnormal findings were in the form of focal epileptiform activity mainly in the left temporal or centrotemporal regions (four patients) and occasionally showing shifting laterality between both sides (three patients). Two of the patients with abnormal encephalogram had autistic regression.

History suggestive of recurrent infection was identified in four patients with mild to moderate autism and four with severe disease. Paternal consanguinity was comparable in both the patients (14.3%) and the control group (16.7%) ($\chi^2 = 0.048$, $P>0.05$) and subjects from both groups did not report any family history of psychiatric disorders. Family history of autoimmune disorders was reported in two patients (9.5%) (Hashimoto's

thyroiditis in one patient with a mild to moderate disease and Psoriasis in another with severe AD) and only one of the control group (4.2%) (Insulin-dependent diabetes mellitus). The difference was not of a statistical significance ($\chi^2 = 0.52$, P> 0.05). Patients with severe autistic disorder did not show significant difference in the family history of autoimmune disorders when compared to those with milder phenotype ($\chi^2 = 0.156$, P> 0.05).

Serum levels of MIF in patients and controls:

Levels of MIF, though lower in the patients [mean± SD: 3.73± 3.9; median (interquartile range) of 2.5 (2.65) ng/ml] were not significantly different from the levels of the control group [mean± SD: 4.1± 3.8; 2.5(3.2) ng/ml] (z=0.79, P> 0.05). Levels of the MIF were significantly lower in patients with severe autistic disorder [median (interquartile range) of 1.85 (1.73) ng/ml] compared to those with mild to moderate disease [4.0 (4.8) ng/ml] (z=2.197, P=0.029) (Fig 1). Correlation of the MIF levels to the different behavioral parameters assessed by the CARS, revealed a negative correlation with the severity of the nonverbal communication parameter derangement (r= -0.49, P=0.042) (Fig 2).

Patients with history of recurrent infections did not show significant difference in MIF levels [median (interquartile range) of 2.5(2.73) ng/ml] compared to those without such history [2.5 (2.8) ng/ml] (P> 0.05)]. There was no significant difference in the levels of MIF in patients with subnormal mentality [median (interquartile range) of 2.35 (2.73) ng/ml] or in patients with abnormal electroencephalogram [median (interquartile range) of 4.0(12.0) ng/ml] compared to the rest of patients [2.8(2.8) and 2.5(3) ng/ml, respectively] (z=0.61 and 0.79, respectively, P> 0.05). Also, patients with autistic regression did not show significant difference in MIF levels [median (interquartile range) of 3.1(2.18) ng/ml] when compared to those with classic- onset autism [3.49(3.05) ng/ml] (z=0.32, P> 0.05).

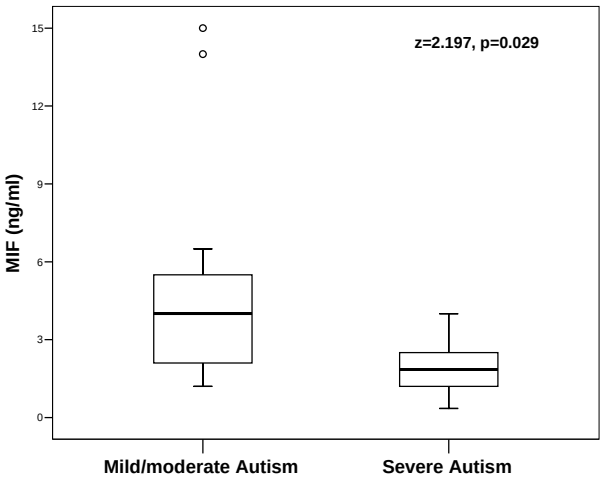

Figure 1. Serum MIF levels in patients with mild to moderate autism and those with severe disorder.

Table 1. Clinical data of the studied population.

	Patients *number (%)*	**Controls** *number (%)*
Type of autism		
Classic –onset	17 (81%)	
Autistic regression	4 (19 %)	
Positive history of seizures	4 (19 %)	
Positive history of recurrent infection	8 (38.1%)	
Positive history of parental consanguinity	3 (14.3%)	4 (16.7%)
Positive family history of neuropsychiatric disorder	0	0
Positive family history of autoimmune disorder	2 (9.5%)	1 (4.2%)
Subnormal intellectual functions (<70)	12 (57.1%)	
Childhood autism rating scale		
Mild to moderate	11 (52.4%)	
Severe	10 (47.6%)	
Positive EEG abnormality	7(33.3%)	

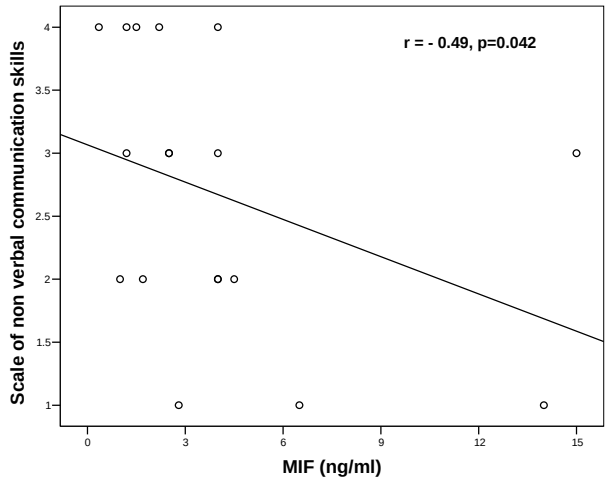

Figure 2: Correlation of serum levels of MIF to the non verbal communication skills severity scale (CARS).

DISCUSSION

There is increasing evidence of immune involvement in AD.[21] Dysregulation of the immune system in particular subtypes of autism is a subject of active investigation in humans and animal models.[10] At present, views of possible immune dysfunction in AD range from conclusions that it may contribute to manifestations of the disorder in some patients[22] to hypotheses that neuroimmunopathogenic responses play a fundamental role in AD.[23] Studies suggest that innate rather than adaptive neuroimmune responses are associated with AD. [24-26]

The results of the present study showed values of MIF that were not significantly different in patients and control groups. However, it was noted that patients with severe disorder showed significantly lower levels of the cytokine than those with milder disease. By comparison, in the only other study assessing MIF in AD, the authors reported a genetic association between functional polymorphisms in the promoter of MIF and autism spectrum disorder- related behaviors, with elevated levels of MIF in the included patients. They proposed an underlying autoimmune basis of the disorder.[27] The difference in our results may be explained on the basis of perhaps another form of polymorphism that may result in decreased levels of MIF in a subset of patients. This may well explain the heterogeneity among AD patients. Whether the reported increased MIF levels in the previous study[27] and also in our subgroup of milder disease, though insignificant, may reflect an increased production of an aberrant nonfunctioning form of the cytokine, and thus may be explained also on the basis of immune dysfunction rather than autoimmune disorder, is beyond the scope of our study. The possible role of derangement of innate immune system has been previously reported. In a study[11] evaluating innate immunity in a subset of children with AD, the investigators assessed the production of proinflammatory and counter-regulatory cytokines by peripheral blood mononuclear cells (PBMCs) in response to agonists of Toll-like receptors (TLRs). The authors noted that peripheral blood mononuclear cells (PBMCs) from AD test group children produced less IL-1β with a TLR7/8 agonist. Other studies reported intrinsic defects of innate immunity with abnormal production of proinflammatory cytokines from cultured PBMCs.[25-26]

Our results and those of other studies might indicate a role of MIF deficiency in development of severe manifestations of the disease. A possible explanation may be through the role the innate immunity plays as the first line, antigen-independent immune defense mechanism. This is achieved by recognizing microbial by-products or those from damaged tissue cells via pattern recognition receptors including TLRs.[28-29] TLR-mediated responses lead to the production of various soluble mediators that can signal the brain.[30-33] Such signaling events help the central nervous system restore autonomic homeostasis and provide inhibitory regulatory signals to prevent excessive immune responses.[30] In further support of this finding is the significant negative correlation between the cytokine and the degree of involvement of nonverbal communication parameter of CARS which is one of the core features of the disorder. The lower the MIF, the worse is the behavioral parameter.

It has been previously noted that a number of autistic children suffer from recurrent infections (typically viral syndromes) accompanied by exacerbations of behavioral symptoms (hyperactivity, temper tantrums, irritability and self-stimulatory behaviors). Immune insult via microbial infection caused by various pathogens appears to counter-act beneficial effects of behavioral, dietary, and other intervention measures in these AD children.[11] The above-described clinical observation was supported by an open-label trial of administration of vancomycin, which resulted in objective, cognitive improvements in autistic children. Furthermore, there was regression of patients' cognitive functions when it was stopped.[35] This further supports the hypothesis that in these AD children, antigen non-specific (innate) immune responses are altered, leading to dysregulated neuro-immune interactions. That our findings did

not show difference in MIF levels between patients with recurrent infections and those without may be explained by the small number of the included group and the need for a more objective evaluation of "recurrent infection," possibly through follow up studies.

It was previously noted that about 20 to 30% of AD parents, report the occurrence of language regression or a developmental plateau associated with loss of sociability in their child between approximately 12 to 30 months of age, usually with no known trigger (autistic regression),[36,8,10] a value that is close to our results. Other neurological abnormalities have been previously described in AD; for example, 30% of children with AD develop epilepsy by adolescence,[37] and an additional group reaching 60% in some studies has subclinical epilepsy, as measured by epileptiform encephalogram, especially during sleep.[38-39] Our results showed that 19% of AD patients suffer from seizures and nearly one third have abnormal epileptiform encephalogram. Both autistic regression and findings of epileptiform encephalogram clearly indicate that there are neurological involvements in AD that affect the development and differentiation of neurons in the brain. Immune dysregulation could result in the generation of localized or systemic inflammation and/or the release of immunomodulatory molecules that could influence, alter, or modify neurodevelopment and/or neuronal function, especially at critical times of development.[10] Interestingly, the presence of low-grade chronic inflammation has been reported in brain tissue of individuals with autism.[9, 24] Our results failed to demonstrate a significant difference in MIF levels in patients with mental retardation or those with abnormal electroencephalogram.

In previous studies on families of affected children, results indicated a higher prevalence of autoimmune disorders like rheumatoid arthritis, lupus, and thyroiditis compared with control families,[40-41] a finding not corroborated in another study.[42] Similarly our results did not show significant difference in the frequency of autoimmune diseases in families of autistic patients compared to the control group. This is in accordance with our results which suggest dysfunction in the innate immune system rather than autoimmune disorder as the underlying pathogenic mechanism.

In conclusion, our findings demonstrated MIF levels that were significantly lower in a subset of patients with severe AD compared to those with milder form and even showed a negative correlation to the nonverbal communicative difficulties those patients experience. These results may indicate a role of the cytokine in the development of severe manifestations of the disorder. Children with severe AD may be less capable of controlling microbial infection in the initial stages, leading to ineffective signaling to the brain. While the extent to which many of the observations discussed herein are involved in the pathogenesis of autism is unknown, it cannot be discounted that immune dysfunction is an epiphenomenon or a consequence of the disease. These findings will require additional study in other samples of probands with AD to determine their replicability. These results also prompt a reconsideration of previous observations and stimulate the investigation of new hypotheses regarding relationships between immune dysfunction and AD. Our results may assist in better defining AD phenotypes, thereby improving the prognosis of behavioral abnormalities and potentially enabling new pharmacologic interventions.

REFERENCES

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 4th edition. Arlington (VA): American Psychiatric Association; 2000. p. 84.
2. Committee on Children with Disabilities. American Academy of Pediatrics: The pediatrician's role in the diagnosis and management of autistic spectrum disorder in children. Pediatrics 2001;107:1221–6.
3. **Keller F, Persico AM.** The neurobiological context of autism. Mol Neurobiol 2003; 28(1):1–22.
4. **Beaudet AL.** Is medical genetics neglecting epigenetics? Genet Med 2002; 4(5):399–402.
5. **London EA.** The environment as an etiologic factor in autism: a new direction for research. Environ Health Perspect 2000; 108 (Suppl3):401–4.
6. **Rapin I, Tuchman RF.** Autism: Definition, Neurobiology, Screening, Diagnosis. Pediatr Clin North Am 2008; 55:1129-46.
7. **Korvatska E, Van de Water J, Anders TF, Gershwin ME.** Genetic and immunologic considerations in autism. Neurobiol Dis 2002; 9:107-25.
8. **Mostafa GA, El-Sayed ZA, Abd El-Aziz MM, El-Sayed MF.** Serum anti-myelin associated glycoprotein antibodies in Egyptian autistic children. J Child Neurol 2008; 23; 1413-8.
9. **Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA.** Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol. 2005; 57:67–81.
10. **Ashwood P, Wills S, Van de Water J.** The immune response in autism: a new frontier for autism research. J Leukoc Biol 2006; 80(1):1–15.

11. **Jyonouchi H, Geng L, Cushing-Ruby A, Quraishi H**. Impact of innate immunity in a subset of children with autism spectrum disorders: a case control study. J Neuroinflammation 2008; 5:52.
12. **Bertoglio K, Hendren RL.** New Developments in Autism. Psychiatr Clin North Am 2009; 32:1–14.
13. **Larson DF, Horak K.** Macrophage migration inhibitory factor: controller of systemic inflammation. Crit Care 2006; 10(2): 138.
14. **Arenberg DA, Bucala R.** Macrophage migration inhibitory factor (MIF). In: Thomson AW, Lotze MT, editors. The Cytokine Handbook. 4th ed. London: Elsevier Science 2003. p. 1037-48.
15. **Roger T, David J, Glauser MP, Calandra T.** MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature 2001; 414:920-4.
16. **Mitchell RA, Liao H, Chesney J, Fingerle-Rowson G, Baugh J, David J, et al.** Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proc Natl Acad Sci USA 2002; 99:345-50.
17. **Oberholzer A, Oberholzer C, Moldawer LL.** Interleukin-10: a complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug. Crit Care Med 2002; 30:S58–S63.
18. **Dalakas MC.** Invited article: inhibition of B cell functions: implications for neurology. Neurology 2008;3,70(23):2252-60.
19. **Schopler E, Reichler RJ, Renner BR.** The Childhood Autism Rating Scale (CARS), for Diagnostic Screening and Classification in Autism. New York, NY: Irvington; 1986.
20. **Terman LM, Merrill MA.** Stanford-Binet Intelligence Scale: Manual for the 3rd Revision Form L-M. Chicago, IL: The Riverside Publishing Company; 1973.
21. **Patterson PH.** Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. Behav Brain Res 2008; Dec 24 [Epub ahead of print].
22. **Ashwood P, Van de Water J.** Is autism an autoimmune disease? Autoimmun Rev 2004; 3(7–8):557–62.
23. **Zimmerman AW, Jyonouchi H, Comi AM, Connors SL, Milstien S, Varsou A et al.** Cerebrospinal fluid and serum markers of inflammation in autism. Pediatr Neurol 2005; 33(3):195–201.
24. **Pardo CA, Vargas DL, Zimmerman AW.** Immunity, neuroglia and neuroinflammation in autism. Int Rev Psychiatry 2005; 17(6):485–95.
25. **Jyonouchi H, Geng L, Ruby A, Zimmerman-Bier B.** Dysregulated innate immune responses in young children with autism spectrum disorders: their relationship to gastrointestinal symptoms and dietary intervention. Neuropsychobiology 2005; 51(2):77-85.
26. **Ashwood P, Schauer J, Pessah IN, de Water JV**. Preliminary evidence of the in vitro effects of BDE-47 on innate immune responses in children with autism spectrum disorders. J Neuroimmunol 2009; 208(1-2):130-5.
27. **Grigorenko EL, Han SS, Yrigollen CM, Leng L, Mizue Y, Anderson GM, et al.** Macrophage migration inhibitory factor and autism spectrum disorders. Pediatrics 2008; 122: e438-45.
28. **Beutler B, Hoebe K, Georgel P, Tabeta K, Du X.** Genetic analysis of innate immunity: identification and function of the TIR adapter proteins. Adv Exp Med Biol 2005; 560:29–39.
29. **Pasare C, Medzhitov R.** Toll-like receptors: linking innate and adaptive immunity. Adv Exp Med Biol 2005; 560:11–8.
30. **Wrona D.** Neural-immune interactions: an integrative view of the bidirectional relationship between the brain and immune systems. J Neuroimmunol 2006; 172:38–58.
31. **Dantzer R.** Innate immunity at the forefront of psychoneuroimmunology. Brain Behav Immun 2004; 18:1–6.
32. **Glezer I, Simard AR, Rivest S.** Neuroprotective role of the innate immune system by microglia. Neuroscience 2007; 147:867–83.
33. **Haddad JJ.** On the mechanisms and putative pathways involving neuroimmune interactions. Biochem Biophys Res Commun 2008; 370:531–5.
34. **Smith DW, Nagler-Anderson C.** Preventing intolerance: the induction of nonresponsiveness to dietary and microbial antigens in the intestinal mucosa. J Immunol 2005; 174:3851–7.
35. **Sandler RH, Finegold SM, Bolte ER, Buchanan CP, Maxwell AP, Vaisanen ML, et al.** Short-term benefit from oral vancomycin treatment of regressive-onset autism. J Child Neurol 2000; 15:429-35.
36. **Fombonne E.** The prevalence of autism. JAMA 2003; 289:87-9.
37. **Volkmar F, Cook EH Jr, Pomeroy J, Realmuto G, Tanguay P.** Practice parameters for the assessment and treatment of children, adolescents, and adults with autism and other pervasive developmental disorders. American Academy of Child and Adolescent Psychiatry Working Group on Quality Issues. J Am Acad Child Adolesc Psychiatry 1999; 38(12 Suppl):32S-54S.
38. **Chez MG, Chang M, Krasne V, Coughlan C, Kominsky M, Schwartz A.** Frequency of epileptiform EEG abnormalities in a sequential screening of autistic patients with no known clinical epilepsy from 1996 to 2005. Epilepsy Behav 2006; 8(1):267-71.
39. **Kim HL, Donnelly JH, Tournay AE, Book TM, Filipek P.** Absence of seizures despite high prevalence of epileptiform EEG abnormalities in children with autism monitored in a tertiary care center. Epilepsia 2006; 47(2):394-8.

40. **Comi AM, Zimmerman AW, Frye VH, Law PA, Peeden JN.** Familial clustering of autoimmune disorders and evaluation of medical risk factors in autism. J Child Neurol 1999;14(6): 388–94.
41. **Sweeten TL, Bowyer SL, Posey DJ, Halberstadt GM, McDougle CJ.** Increased prevalence of familial autoimmunity in probands with pervasive developmental disorders. Pediatrics 2003;112:e420.
42. **Mouridsen SE, Rich B, Isager T, Nedergaard NJ.** Autoimmune diseases in parents of children with infantile autism: a case-control study. Dev Med Child Neurol 2007;49(6): 429–32.