Blood-based markers in autism spectrum disorders

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Abstract
A definitive test that is universally accepted for accurately diagnosing Autism Spectrum Disorders (ASD) has yet to be developed. Because ASD results from a combination of genetic susceptibility and environmental factors such as infections, dietary proteins, xenobiotics, and gut dysbiosis, we measured biomarkers in the blood of healthy controls and patients with ASD and Crohn's disease. Using ELISA methodologies, we first measured antibodies against gluten and non-gluten proteins of wheat, α- and β-casein, heavy metals (ethyl mercury, aluminum hydroxide, aluminum phosphate), bisphenol A, artificial food colorants, and glyphosate. These chemicals or their metabolites can affect the gut microbiome and induce overproduction of bacterial toxins, which may result in barrier breakdown. Therefore, we also measured antibodies against LPS, bacterial cytotoxins, occludin/zonulin, and vinculin. Furthermore, since bacterial toxins can damage both the gut barrier and blood-brain barrier, we also measured antibodies against claudin-5, S100B, glial fibrillary acidic protein, myelin basic protein, cerebellar peptide and synapsin. Compared to controls, ASD groups had much higher levels of antibodies against food antigens, chemicals that form neo-antigens with human tissue, tight junction proteins, and neural cells. Regarding cellular immunology, we also measured pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-17; all were found to be significantly elevated in ASD patients. Finally, the regulatory cytokines such as IL-10, TGF-β and regulatory T cells were measured in comparison with controls and were found to be lower in subjects with ASD. The results of this study indicate that a dysregulated immune system, including overproduction of antibodies and inflammatory cytokines with down-regulation of regulatory T cells, plays a role in autism. The development of biomarkers such as those used in this study is essential for improving the diagnosis and management of ASD.

Key words Autism Spectrum Disorders, body burden, neuroautoimmunity, food color, blood-brain barrier, synapsin, cerebellar

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1. Introduction

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders that are characterized by impairments in communication and social interaction.[1] The prevalence of autism currently in the US is reported to be 1 in every 45 children, the majority (75%) of them being boys.[2] This reported increase in the prevalence of autism compared to a decade ago has triggered vigorous debate as to whether this increase is merely a consequence of definition, greater awareness, improved diagnosis and reporting, or does the trend actually reflect a true rise in incidence?[3,4] Genetic factors, deletion, variation in copy number and other genetic abnormalities may all be linked to autism, but the upward trend in reported prevalence can not be explained by the numerous candidate loci on chromosomes 7q, 15q, and 2q.[5,6] Despite the rapid advances in genetics, presently only about 10% of autism cases can be explained purely by the genes.

The clinical and epidemiological aspects of autism, such as the wide heterogeneity in clinical presentation, the occurrence of sporadic cases, and the discordant development of ASD in monozygotic twins suggest that early exposure to environmental factors play a role in the development of ASD.[7] We hypothesize that some environmental immunogens not only impact the susceptibility gene to autism but exert their effect on the gut, the immune system, and the nervous system during the critical early developmental stage of life. Identification of environmental insults, gut and brain barrier dysfunction and neuroautoimmunity may play a role in the treatment of children suffering from ASD.

Although genetic studies of ASD are well-established, research on the role of environmental triggers in ASD is relatively new.[8,9] Many researchers, as cited in this paper, have embarked on correlating specific environmental factors to the gut and brain pathogenesis of ASD. Although, presently, a single blood test is not available for the early detection of autism, the identification of biomarkers based on reactivity to food, toxic chemicals, infectious agents, barrier dysfunction and autoantibodies, provides the strategy of targeting these factors for therapeutic interventions.

1.1. Testing for antibodies to dietary proteins.

Many family members of children with ASD have come to know very well that the afflicted often overreact to many food antigens, especially wheat and dairy. In fact, studies have shown that some autistic behaviors improve when the child is put on a gluten- and dairy-free diet.[10] One of the biggest problems with food immune reactivity tests is that the foods used in the panels do not reflect the manner in which the patient consumes the food. Research has shown that heating food above 118˚F changes the protein structure, which changes the antigenicity of the food.[11] Simply cooking a food can make it more antigenic to one patient, and less antigenic to another. Testing combined food proteins is also important so as to reflect the real-world culinary experience. When combining food proteins, the finished food may be more antigenic. For example a potato fried in

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canola oil may be more antigenic to a patient than a plain baked potato, or compare reactivity to a raw egg versus a hard-boiled egg.[11] Unfortunately, most laboratories only assess immune reactivity to raw food proteins.[12]

Another important factor for food immune reactivity testing to have is antigen purity. Purification of antigens prior to coating the ELISA plates is a vital step for insuring specific and sensitive results. Unpurified food antigens may contain additional immunogens such as bacteria, fungi, pesticides, insect debris and unbound metals.[12] The use of these unpurified food antigens contributes to the problem of false positive or false negative test results. A test is only as good as the purity of the antigen used.

1.2. Immune reaction to toxic chemicals, heavy metals, aluminum, formaldehyde, bisphenol, food coloring and glyphosate.

Rossignol, *et al*. completed a systematic review of publications assessing chemical exposure as a trigger for ASD; the authors concluded that only mercury was consistently found in ecological studies.[13] However, the studies included in the article looked for levels of chemicals in urine, hair, blood, tooth or brain. Levels of chemicals indicate exposure, but do not indicate body burden of chemicals, which is why many of these studies cited could not find a significant difference between ASD and control groups. The only safe way to assess body burden of chemicals or heavy metals is to measure antibodies to the chemicals/metals bound to human tissue. When a chemical/metal binds to human tissue, it becomes a body burden.[14,15] The immune system reacts against the chemical and produces antibodies against both the chemical and the tissue to which it is bound. The healthcare practitioner needs to know what remains in the body, and if the body is launching an immune response. Koch and Calafat[16] summarized the issue in their review article, stating that the levels cannot reveal whether or not the patient has reached a toxic level, as 'high-levels' can be tolerated by some patients and 'low-levels' can be a burden contributing to disease pathogenesis. Assessing levels of chemicals continues to be common practice; however, the best practice is to measure antibody production against the chemicals, such as mercury, that covalently bind to human tissue antigens.

Vaccinations were once targeted as the one trigger of ASD. Several studies as reported in a review and meta-analyses[9] concluded that there was no increased risk for ASD in vaccinated children. Offit, *et al*. published a paper discussing the capabilities of the neonate's immune system that assured parents a baby's immature immune system is capable of handling "an enormous number of antigens," even those infants with compromised immune systems.[17] Another review article published by Pediatrics stated that some studies showed a risk for adverse events when vaccinating immunodeficient children.[18] Eibl and Wolf recommend using intravenous immunoglobulin treatment prior to vaccinating adults with immune deficiencies.[19] Despite this, ethyl mercury, merthiolate or thimerosal, due to anti-bacterial and anti-fungal properties, is widely used in fish farming, vaccines and many medications.[20] The organic mercury

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compound that is currently used in flu vaccines, such as those given to pregnant women, is thimerosal. The potential for health hazards with thimerosal include autoimmunity and neuroautoimmunity. This is due to the fact that after tissue absorption, ethyl mercury binds to the thiol group in cysteine and forms a complex with various proteins, such as enzymes, albumin, hemoglobin and others.

Aluminum is the third most common naturally occurring element after oxygen and silicone. It is found in plants, soil, water, and, unfortunately, is added to a variety of foods including baby formulas. Tangle formation and neuronal protein and nucleic acid cross-linking by aluminum can result in both humoral- and cell-mediated immune response against both neuronal proteins and aluminum, which, on its own, after binding to human tissue, may act as an antigen.[21] As an antigen, it may result in antibody production against aluminum bound to tissue and neuronal cell proteins.

Formaldehyde and glutaraldehyde are common disinfecting chemicals with wide usage in other industries such as furniture and cosmetics, allowing for daily exposure to these two chemicals. Like many other chemicals, formaldehyde covalently binds to mammalian tissue, inducing antibody production against formaldehyde and self-tissue. Based on this mechanism of action, as early as 1988 the senior author already conducted tests that reported the presence of formaldehyde antibodies in samples from residents of mobile homes.[22]

The majority of children are exposed to bisphenol-A (BPA) through the use of plastic bottles and pacifiers. Most clinical laboratories measure levels of BPA, or its metabolites, in urine. Because greater than 90% of the population in the USA has high levels of BPA in urine, this measurement is valuable only as an indication of BPA exposure. What isn't assessed is whether or not the BPA exposure has pathological consequences. For this reason, researchers looked at the metabolites of BPA in children with ASD in order to find an association between BPA exposure and autism. By examining the percentage of bound BPA in ASD and finding that it was 15 times higher than in controls, it was concluded that there was an association between BPA exposure and ASD.[23]

Artificial food colorings, though known to cause DNA damage, adverse effects on the liver and kidneys, and have carcinogenic properties, have not been restricted. Instead, they have actually been increased in use for a growing number of foods over the last 50 years.[24-27] Food colorings are generally ionic and thus they interact strongly with proteins to form covalent bonds.[28]

Glyphosate or *N*-(phosphonomethyl)glycine is one of the most widely used herbicides in the world. It is applied to the leaves of plants to kill weeds, especially broadleaf weeds and grasses that compete with crops.[29] Due to the increased use of genetically engineered crops such as soy, corn, canola, sugar beets and alfalfa, the application of glyphosate in the US as a weed killer has grown enormously. The industry assures the consumers that glyphosate is practically nontoxic, since only about 2% of ingested glyphosate is metabolized into aminomethylphosphonic acid (AMPA), and the other 98% enters the blood stream and is

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eliminated through the urine. This claim for the safety of glyphosate is based mainly on very short-term studies on rodents who have shown non-significant toxicity.[30] However, different studies involving life-long exposure with rodents demonstrated glyphosate suppression of cytochrome P-450 enzyme, liver and kidney dysfunction, and even increased risk of cancer.[31,32]

The mechanism of action of glyphosate in plants is associated with the disruption of the Shikimate pathway, the seven-step metabolic route used by bacteria, fungi, algae, plants and some protozoan parasites.[33] It is completely absent in all animals, but this pathway is present in the gut microbiome. An imbalance of the gut microbiome, perhaps due to the loss of some of our original ancestral bacterial species and/or environmental triggers, has been shown to play a crucial role in almost every single autoimmune disease, including inflammatory bowel disease, celiac disease, autism, other autoimmunities, cancer, and neurodegenerative disorders.[34-38]

1.3. Antibodies to bacterial toxins, gut and blood-brain barriers.

A review of the literature reveals that infectious agents have long been suspected of playing a role in ASDs.[39] Pathogens can enter the body through the intestinal tract. Although ASD is known as a disorder of the brain, research indicates that the majority of ASD patients also suffer from gastrointestinal disorders such as gut dysbiosis, irritable bowels and enhanced intestinal permeability. Gut dysbiosis, with or without increased intestinal permeability, allows for the transfer of bacterial toxins into the bloodstream. Chronic gut disorders can lead to increased intestinal permeability or "leaky gut," which can perpetrate the invasion of undigested food proteins, bacterial toxins, mucosal pathogens and intestinal barrier tissue.[40-42]

Chronic gut dysbiosis, inflammation from multiple food immune reactivity, stress and body burden of chemicals are all known to break down intestinal barrier tissues. As intestinal barrier structures are broken, tissue proteins enter the bloodstream where they can ignite an immune response against them.[43-51] Once there is a breach in the intestinal barrier, the blood-brain barrier (BBB) is at risk for destruction.[52-56] Although a few studies found no association between BBB and neurological tissue antibodies in ASD,[57,58] others found a strong correlation.[59-64]

1.4. Cytokine production

Cytokines play a very important role in many aspects of inflammation and immunity, particularly in neuroautoimmunity. Immune abnormalities including low NK cell activity and abnormally low or very high levels of particular cytokines have been observed in our laboratory as well as by other investigators.[65-68] For example Jyonouchi *et al*.[69] demonstrated that abnormal proinflammatory and regulatory cytokine production was associated with ASD and developmental regressions. Therefore, the measurement of lymphocyte subpopulation, with particular emphasis on $CD4^+CD25^+$ $FoxP3^+$ cells, T-cell function, B-cell function, NK cell activity, and cytokine production is very important in ASD.

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2. Materials and Methods

The following proteins, peptides, and xenobiotics were used: wheat, α-gliadin, γ-gliadin, ω-gliadin, glutenin, gluteomorphin, serpin, CXCR3-binding gliadin, purinin, wheat germ agglutinin (WGA), α-casein and β-casein as food antigens; ethyl mercury, aluminum hydroxide, aluminum phosphate, formaldehyde, glutaraldehyde, bisphenol A, tartrazine, allura red, and glyphosate bound to human serum albumin (HSA) as chemical immunogens; bacterial cytolethal distending toxin (CDT), lipopolysaccharide (LPS), LPS binding proteins (LBP), and tight junction proteins such as occludin/zonulin and vinculin; and blood-brain barriers such as claudin-5, S100B and glial fibrillary acidic protein (GFAP); and neural antigens, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), cerebellar peptide, Purkinje cell, and synapsin. Immunogens were either purchased from Sigma-Aldrich® (St. Louis, MO, USA) or synthesized by Bio-Synthesis® (Lewisville, TX, USA). For the measurement of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, transforming growth factor (TGF)-β, IL-10, and IL-17, ELISA kits purchased from R&D were used. For the determination of regulatory T cells, monoclonal antibody for the detection of $CD4^+CD25^+$ FoxP3 was purchased from Becton Dickenson.

The binding of the herbicide glyphosate to HSA was done according to the methodology described by Yue *et al*. in 2008,[70] and the binding of other chemical haptens to HSA was done according to the methodology used by Vojdani *et al*. in 2015.[15]

2.1. Blood samples from patients and controls

Sera from 50 subjects diagnosed with autism (33 males and 17 females), 3-14 years of age, and sera from 50 patients (50/50% male-female, 18-65 years of age) diagnosed with Crohn's disease and confirmed by anti-*Saccharomyces cerevisiae* IgA ELISA kit (INOVA, San Diego, CA, USA) were sent by healthcare practitioners to our laboratory; for comparison, 50 healthy matched controls (Innovative Research Inc., Southfield, MI, USA) with negative anti-nuclear antibody titers and no detected autoimmune diseases were included. Donor samples were verified to not exhibit health complaints at the time of the blood donation and each specimen tested negative according to FDA guidelines for the detection of hepatitis B surface antigen, antibodies to HIV, antibodies to hepatitis C, HIV-1 RNA, hepatitis C RNA and syphilis. No additional information about the donors in regards to work/home environments or possible exposure to environmental insults was provided. An additional 200 samples (3:1 ratio male to female, 2-15 years of age) from patients with a diagnosis of autism were sent to our lab by different clinicians. The clinical diagnosis of autism was made according to the DSM IV and/or ICD-10 criteria established by the American Psychiatric Association (Washington, DC). Blood samples from 113 children ages 5–15 who came to our laboratory for allergy testing were used as controls. Only individuals with IgEb antibody less than 50 IU and who were completely negative for the tested environmental allergens were used.

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2.2. Measurement of antibodies

Serological antibodies were measured using enzyme-linked immunosorbent assay (ELISA). ELISA was performed on sera from patients and controls as described below. Antigens and peptides were dissolved in methanol at a concentration of 1.0 mg/ml, then diluted 1:100 in 0.1 M carbonate-bicarbonate buffer, pH 9.5 and 100 μl were added to each well of a polystyrene flat-bottom ELISA plate. Plates were incubated and washed according to ELISA standards. (For a full description of ELISA testing methods, see Vojdani.[1-7,10-12,14,15,71,72]) The optical density of each well was read at 405 nm by means of a microtiter reader. To detect non-specific binding, several control wells contained all reagents except human serum, or wells were coated with different tissue antigens and other reagents were used.

3. Results

3.1. Antibody against dietary proteins

In the search for usable biomarkers of ASD, we used ELISA methodology for the determination of antibodies, first against dietary proteins, then against xenobiotics, bacterial toxins, and tissue antigens. Because of the high diversity of wheat phenotypes and the variety of antigenic determinants, we measured IgA antibodies against various wheat proteomes in 50 patients with ASD and 50 Crohn's patients, and compared them to 50 healthy subjects. The results are summarized in Figure 1. Clearly the ASD and Crohn's groups showed much higher levels of IgA antibody against α-gliadin, glutenin, gluteomorphin, CXCR3-binding gliadin peptide, and WGA than healthy controls (p<0.001). The mean of ELISA indices plus two standard deviations (2SD) were calculated for all three groups. Compared to controls, these indices were the most significant for CXCR3-binding gliadin peptides, glutenin, and wheat ge rm agglutinin (WGA) in ASD as well as in Crohn's disease, with patients $p < 0.0001$ (Figure 1).

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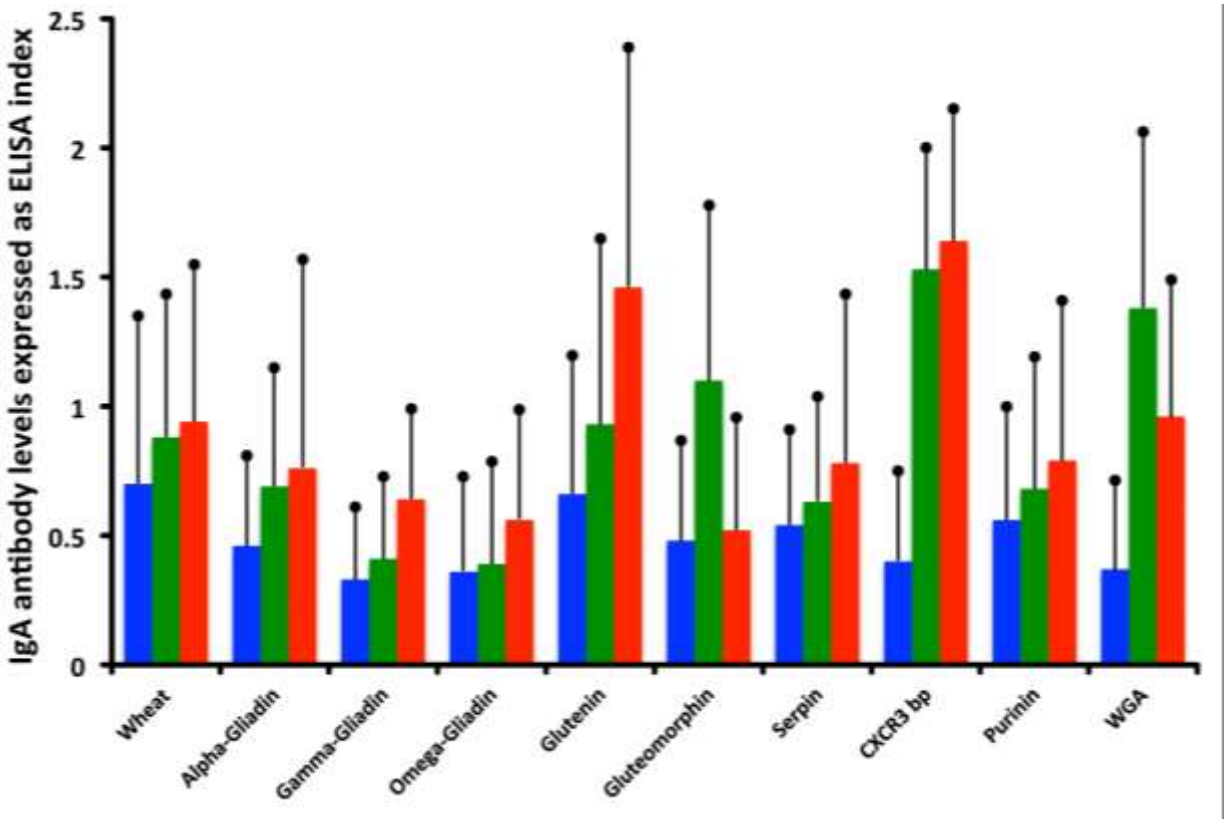

Figure 1. IgA antibodies against various wheat proteomes in 50 ASD patients ■, 50 Crohn's patients ■, and 50 healthy subjects ■. IgA antibody is significantly higher in both ASD and Crohn's disease when measured against CXCR3-binding gliadin peptide, gluteomorphin, and WGA.

Based on the mechanism that chemicals bind to human tissue, thus forming neo-antigens, antibodies were measured against ethyl mercury-HSA, aluminum-HSA, and formaldehyde-HSA in ASD and in controls. The results for IgG, IgM and IgA antibodies against ethyl mercury against 50 ASD patients and 50 healthy controls is shown in Figure 2. At 2SD above the mean, the ASD group exhibited mercury-HSA antibody in up to 22%, compared to controls with an elevation of 9-14%.

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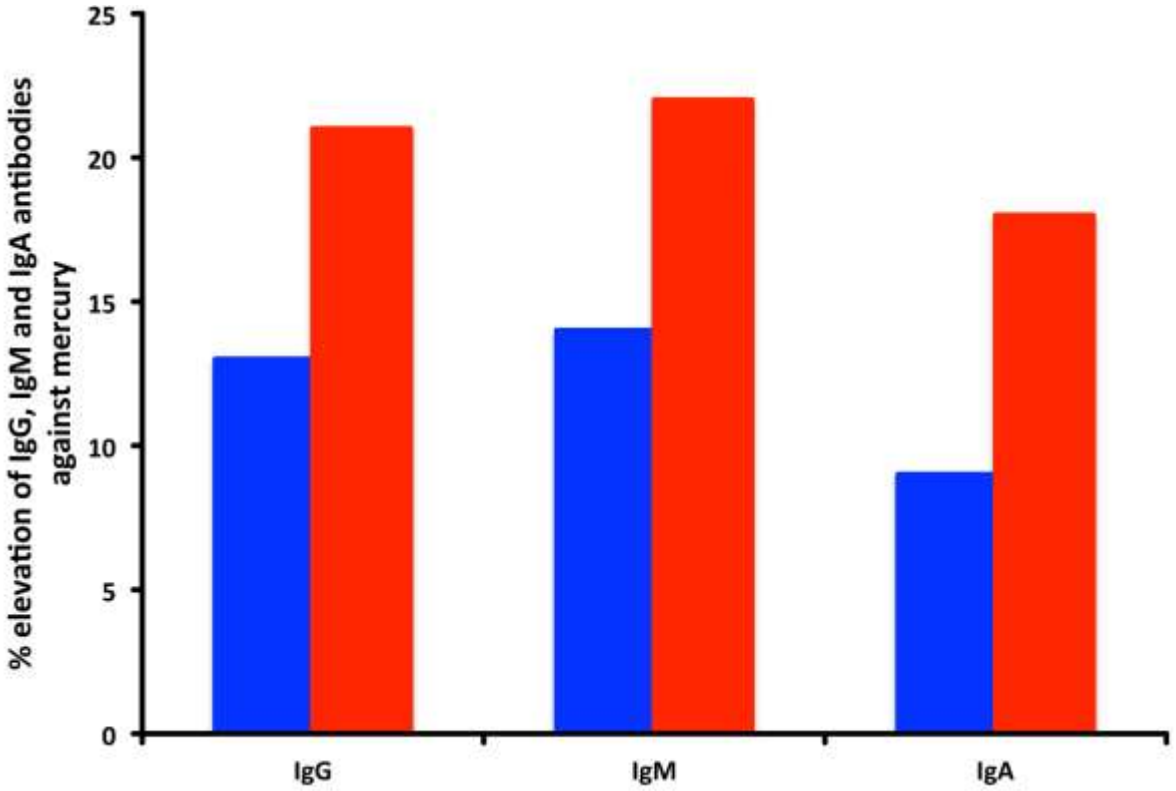

Figure 2. Percentage elevation of IgG, IgM and IgA antibodies against ethyl mercury in 50 healthy controls ■ and in 50 children with ASD ■.

3.2. Antibody against chemicals bound to HSA

We measured IgG antibody against aluminum hydroxide and aluminum phosphate bound to HSA in 50 healthy subjects and 50 ASD children. Results are shown in Figure 3. Although significant levels were found in both healthy (20% and 22%) and ASD (26% and 27%) groups for reactivity to aluminum hydroxide and aluminum phosphate respectively, percentages were higher in the ASD groups. Furthermore, about 80% of the individuals with high levels of antibodies reacted simultaneously against both aluminum hydroxide and aluminum phosphate bound to HSA.

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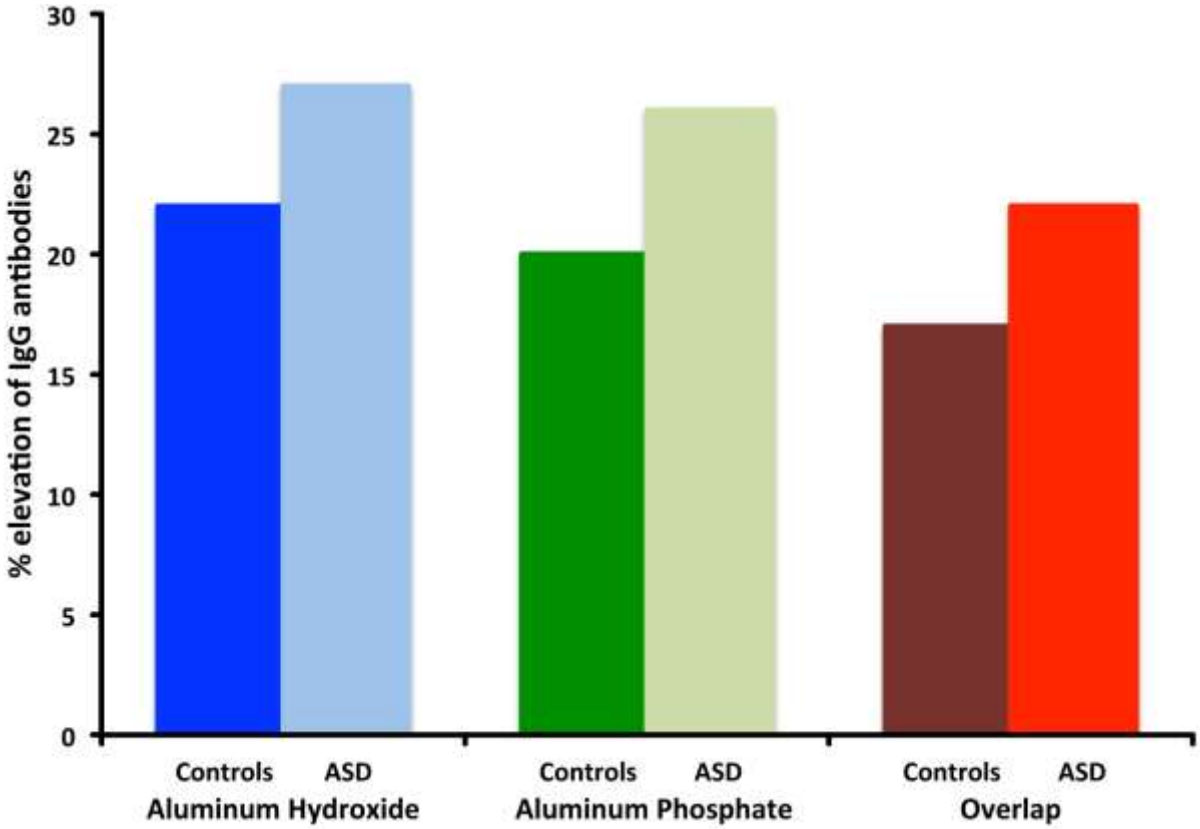

Figure 3 Percentage elevation of IgG antibody against aluminum hydroxide and aluminum phosphate bound to HSA in 50 healthy subjects and 50 ASD children..

We measured antibodies against formaldehyde + glutaraldehyde in 50 healthy subjects and found that 8% produced IgG and 13% produced IgM antibodies. When we extended this antibody measurement to the sera of 50 children with ASD, we found a significantly higher percentage of ASD patients showed elevation in IgG (17%) and IgM (21%) against these chemicals bound to HSA with $p < 0.001$ (Figure 4).

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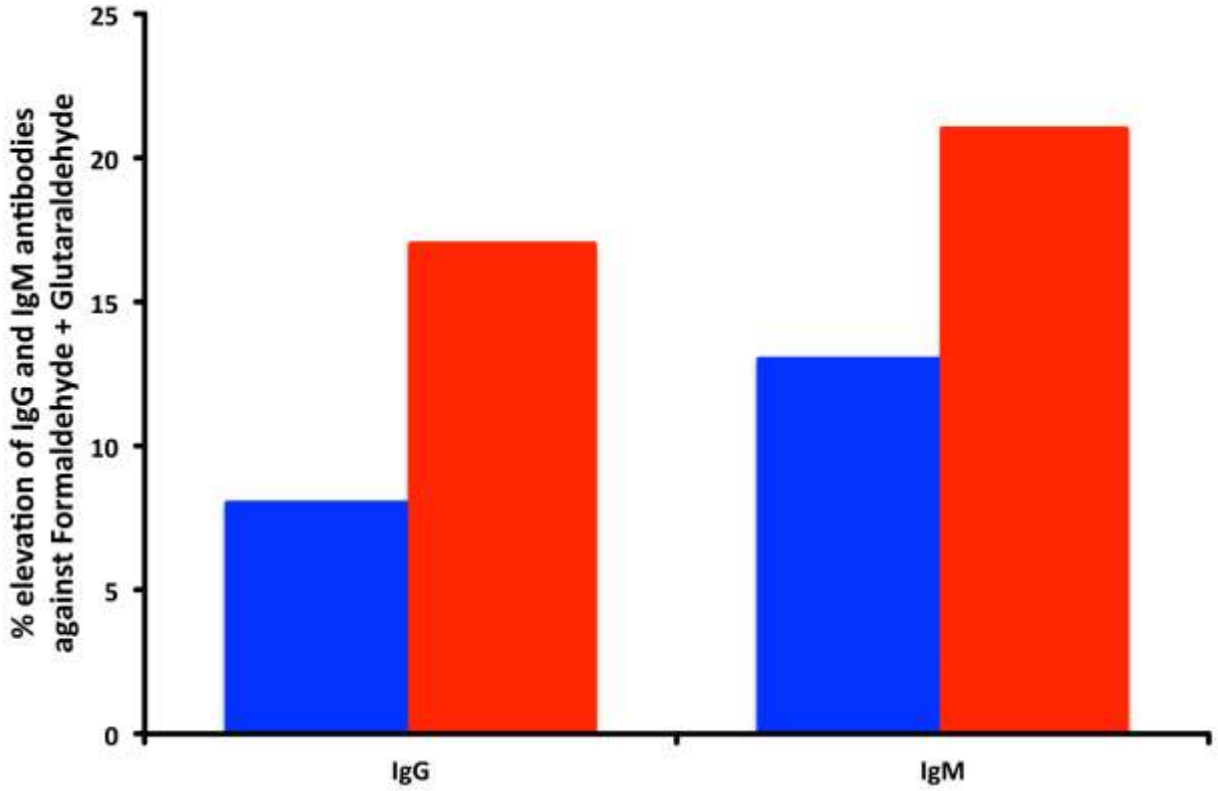

Figure 4. Percentage elevation of IgG, IgM and IgA antibodies against formaldehyde + glutaraldehyde in 50 healthy subjects ■ and in 50 children with ASD ■.

Because BPA and its metabolites can bind to different proteins, we measure antibodies against BPA-HSA adduct in 50 healthy subjects and in 50 children with ASD. Analysis of the data (Figure 5) showed that at 2SD above the mean of the control, up to 17% had elevations in IgG, IgM or IgA antibodies against BPA-HSA. In ASD this percent elevation was significantly higher than in controls, p <0.001, up to 29% of children with ASD had very high IgG, IgM or IgA antibody against BPA-HSA. Meaning in ASD, BPA, or its metabolites, manage to bind to tissue antigens more than in controls. Therefore, measuring BPA-HSA adduct antibody could serve as a biomarker in a subgroup of children with ASD.

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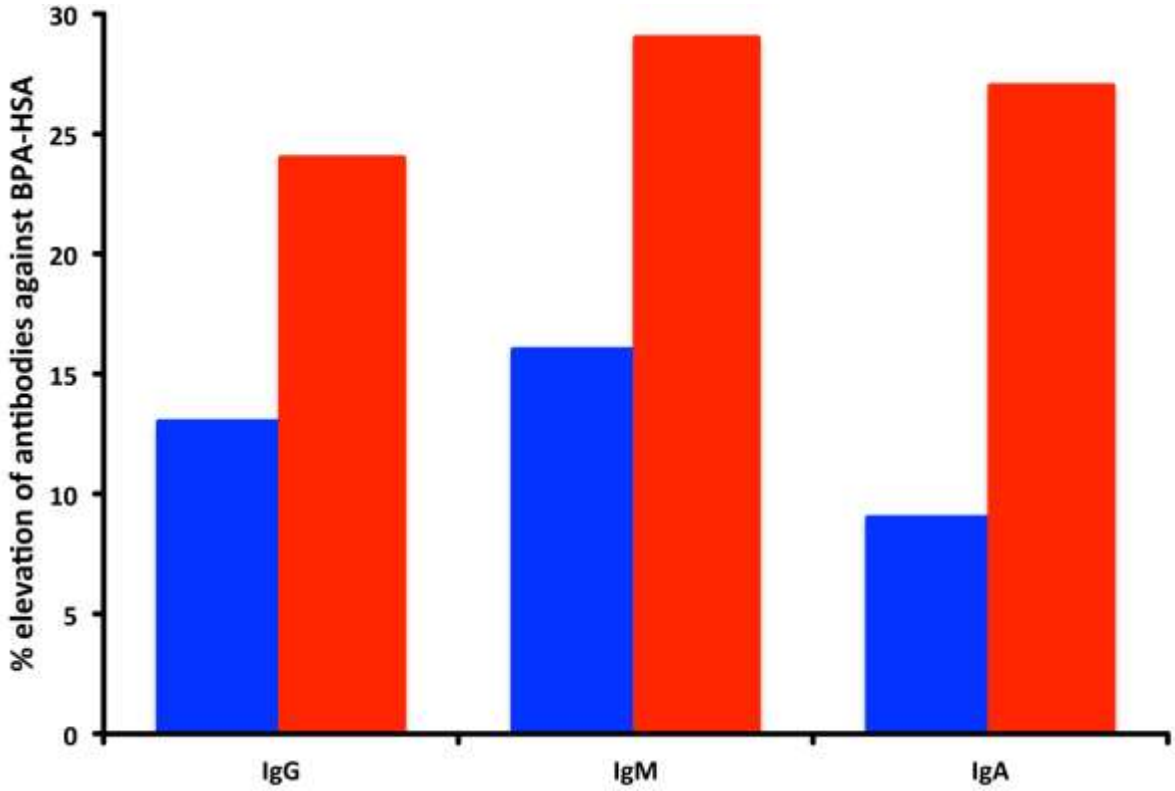

Figure 5. Percentage elevation of IgG, IgM and IgA antibodies against BPA-HSA adduct in 50 healthy subjects ■ and in 50 children with ASD ■.

Just as with aluminum, humans are exposed daily to food colorants in their environment. When we measured IgG antibody against the mixture of artificial food coloring at 2SD above the mean, 10 out of 50 control subjects (20%), versus 13 out of 50 ASD (26%) showed a significant elevation in IgG antibody against protein-colorant complexes (Figure 6).

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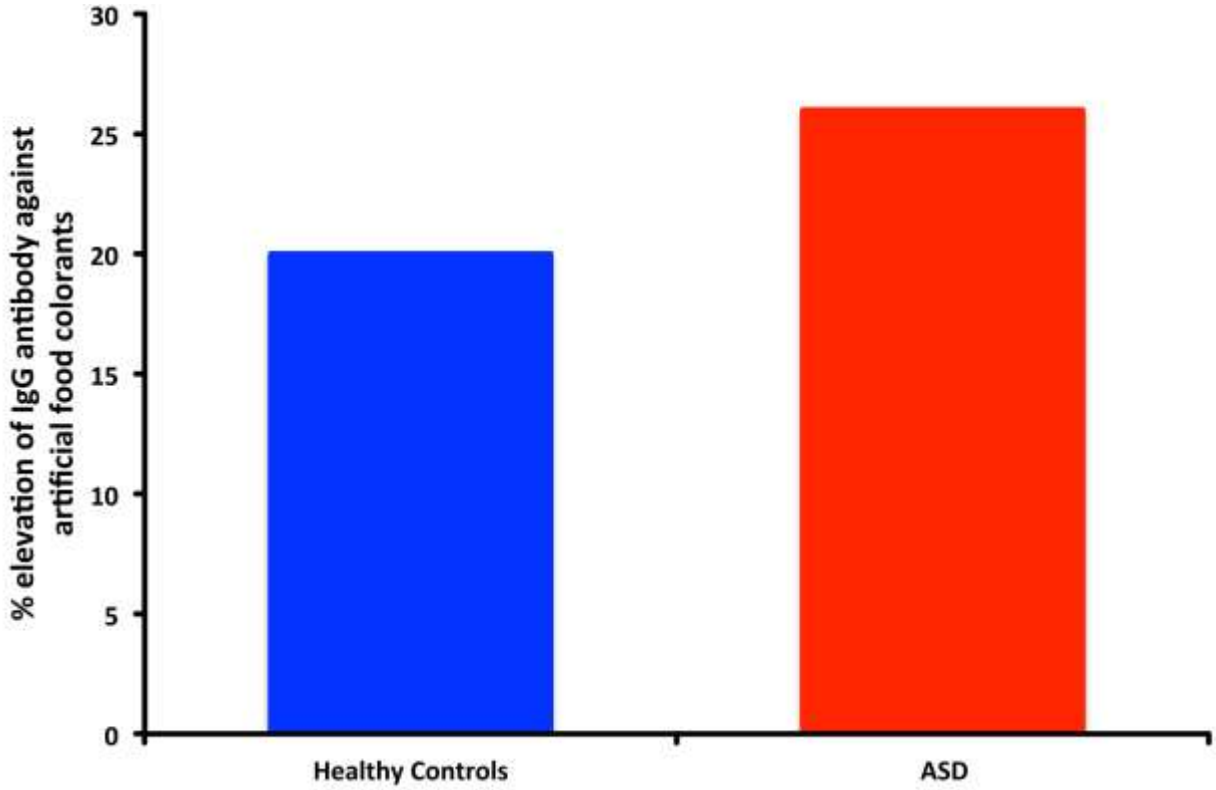

Figure 6. Percentage elevation of IgG antibody against artificial food colorants in 50 healthy controls ■ and in 50 children with ASD ■.

The interaction or binding of glyphosate to HSA enabled us to measure antibody to glyphosate-HSA in the blood of 50 controls and 50 patients with ASD. Results depicted in Figure 7 show that at 2SD above the mean, 19% of controls and 28% of the ASD samples showed a significant elevation in IgG antibody. The mean ELISA index for controls was 0.55, while for the ASD group it was 0.93 ($p < 0.001$).

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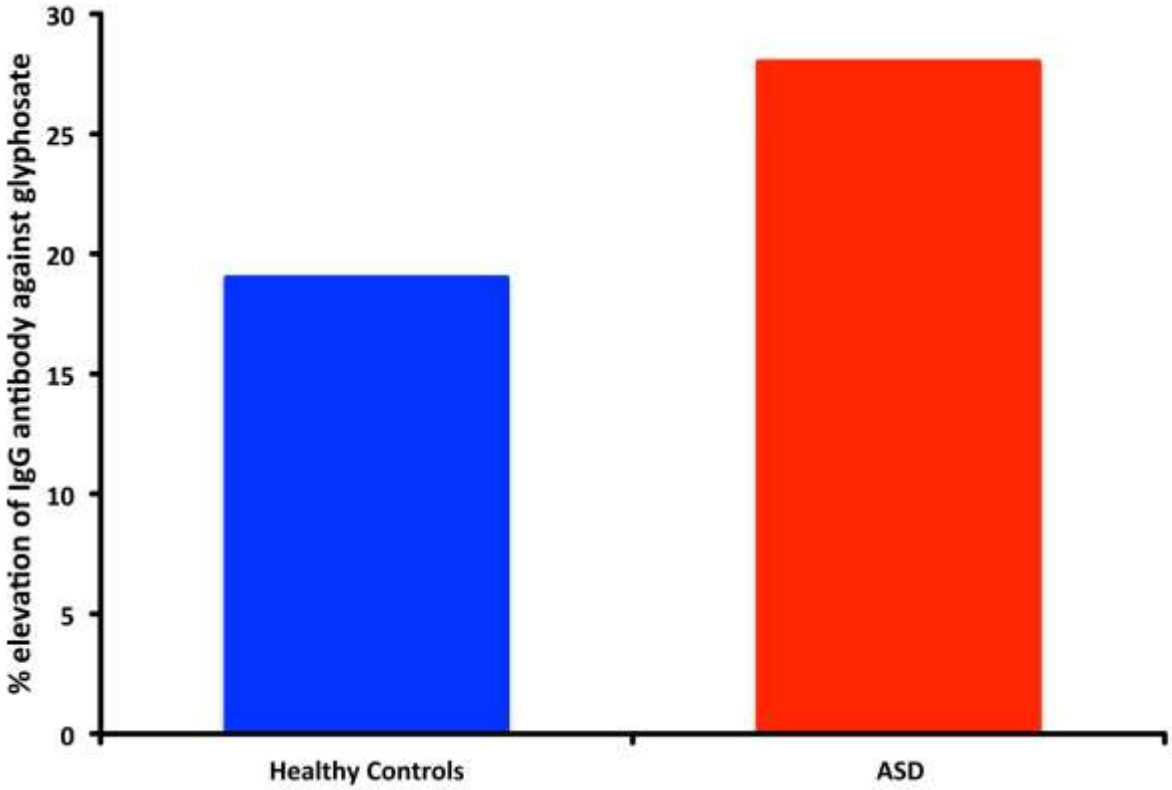

Figure 7. Percentage elevation of IgG antibody against glyphosate-HSA in 50 healthy controls ■ and in 50 children with ASD ■.

3.3. Antibodies against bacterial toxins, tight junction proteins and neuronal cell antigens

We measured antibodies against LPS, bacterial cytotoxins (CDT), LPS binding protein (LBP), occludin/zonulin, and vinculin in 50 healthy subjects and 50 ASD patients (Figure 8). The results showed a significant elevation in IgG antibody index against bacterial toxins and tight junction proteins in the samples from ASD patients ($p < 0.0001$). These results indicate that chemicals, by changing the integrity of the human microbiome, can induce the release of bacterial toxins, which may be followed by breaches in the body barriers, such as the gut barriers and BBBs.

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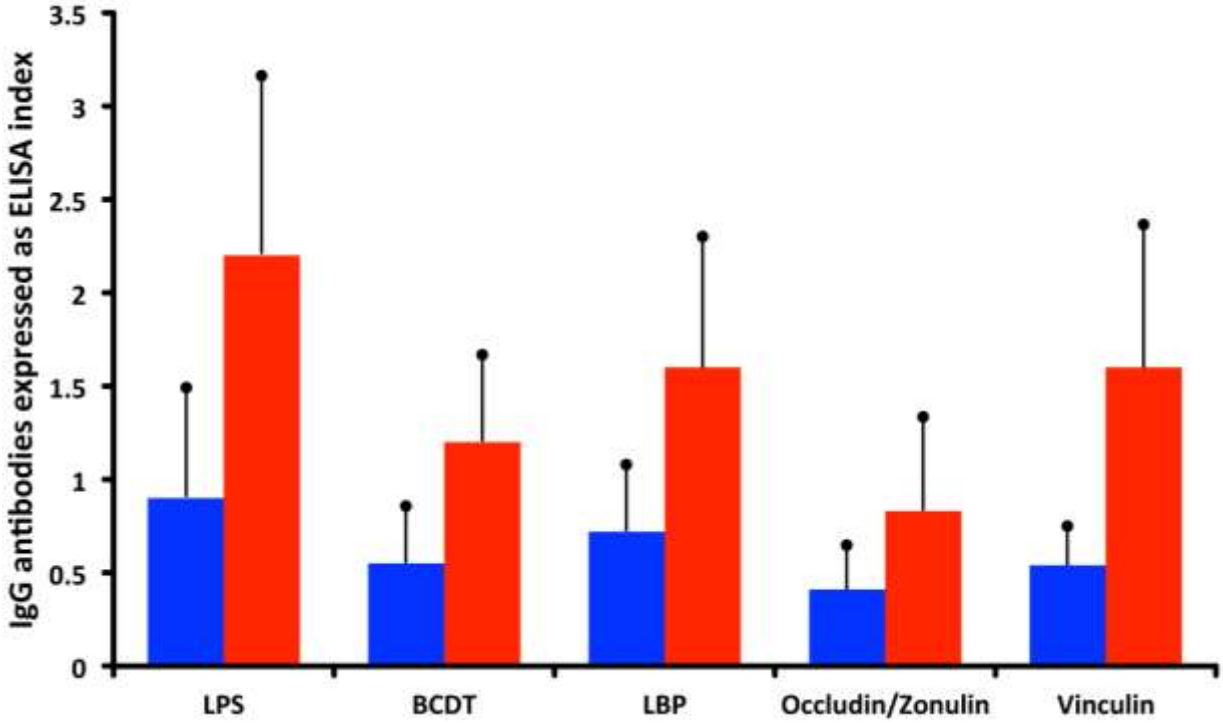

Figure 8. Levels of antibodies against LPS, bacterial cytotoxins (BCDT), and LPS binding protein (LBP) in 50 healthy subjects ■ and 50 ASD patients ■.

We also measured IgG antibodies to BBB proteins in 50 healthy subjects and 50 ASD patients (Figure 9). When compared to healthy subjects, children with ASD produced significantly higher levels of IgG antibodies against claudin-5 (an adherens junction protein), S100B, an astrocytic antigen, glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), cerebellar peptide, Purkinje cells, and synapsin. Referring back to Figure 9, we found that a subgroup of children with ASD produced significantly higher levels of IgG antibodies against neural antigens. This elevation in brain-specific antibodies against all tested BBB proteins and neural-specific antigens in a subgroup of ASD patients resulted in a mean ± SD ELISA ODs much higher than healthy subjects, with $p < 0.0001$.

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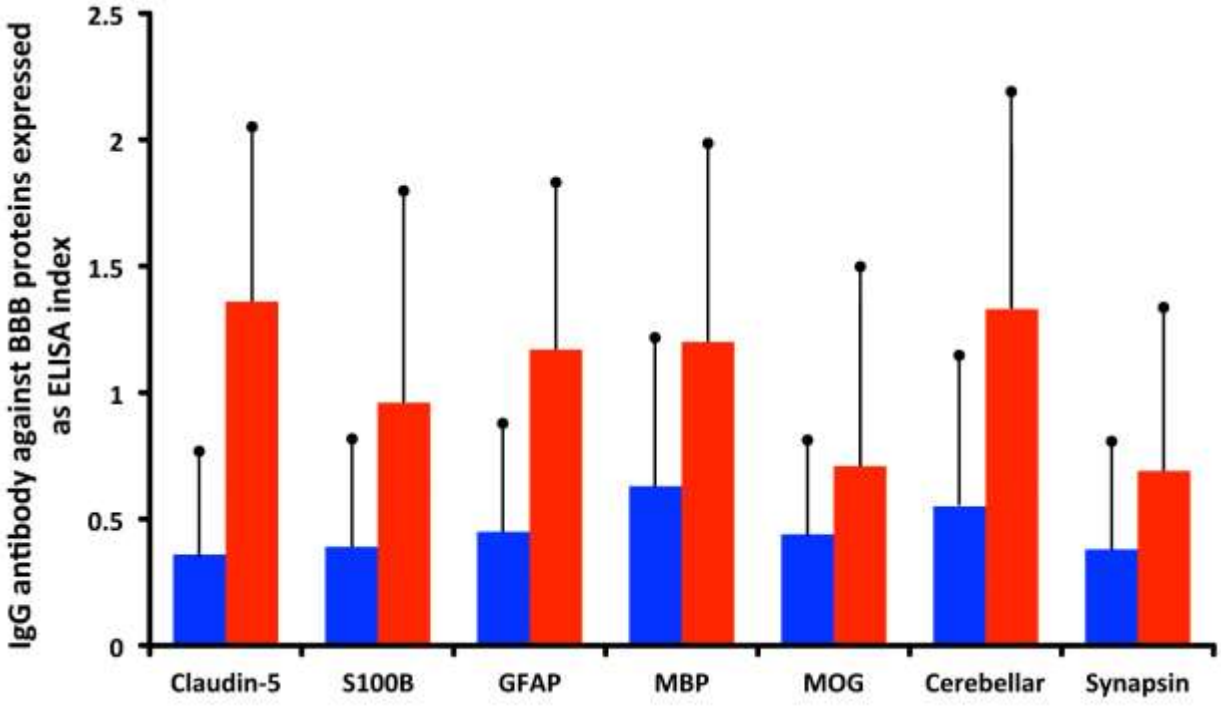

Figure 9. Levels of IgG antibodies against BBB proteins in 50 healthy subjects ■ and 50 ASD patients ■.

3.4. Measurement of cytokines and regulatory T cells

In our lab, we cultured blood samples from 200 ASD children and 113 healthy controls and measured the level of produced cytokines after 48 hours of exposure to T-cell mitogen (PHA). We found that in a subgroup of ASD children, the levels of IL-10 and TGF-β were much lower than in controls (p <0.001), while the levels of IL1-β, IL-6 and TNF-α were significantly higher than the control group (Figure 10). Th17 is another subset of T-helper cells that produce IL-17A and have been suggested to be mediators of inflammation associated with various autoimmune diseases, including rheumatoid arthritis and multiple sclerosis.[73]

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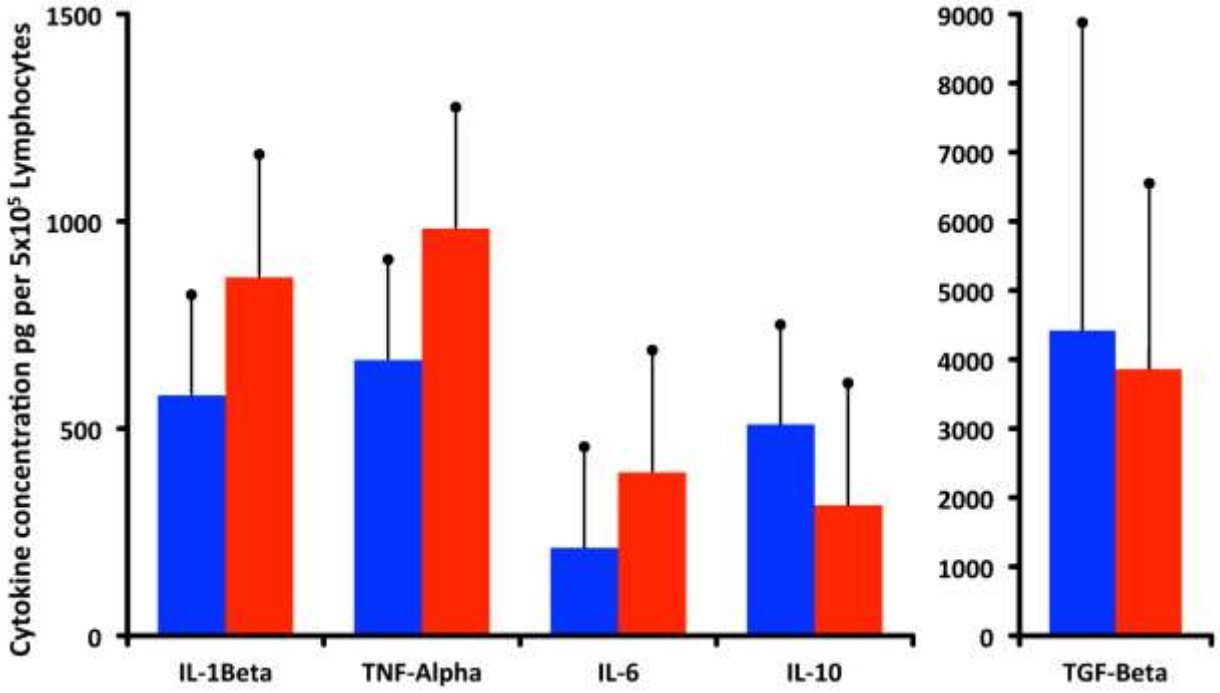

Figure 10. Cytokine production 48 hours after culture of lymphocytes in the presence of T-cell mitogen (PHA). Data was calculated from 113 controls ■ and 200 children with ASD ■.

Because a subgroup of children with ASD suffer from inflammation and autoimmunity, particularly, neuroautoimmunity, we measured IL-17A production in 113 healthy controls and 200 children with autism. Similar to other cytokine assessments, IL-17A was measured after 48 hours of lymphocyte culture in the presence of PHA. Results, summarized in Figure 11, showed that in comparison to controls, a subgroup of ASD children had a significant elevation in IL-17A production (p <0.001). Furthermore, elevation in IL-17A production conversely correlated with low percentage of $CD4^+CD25^+$ regulatory T-cells in ASD (p <0.01) (Figure 12).

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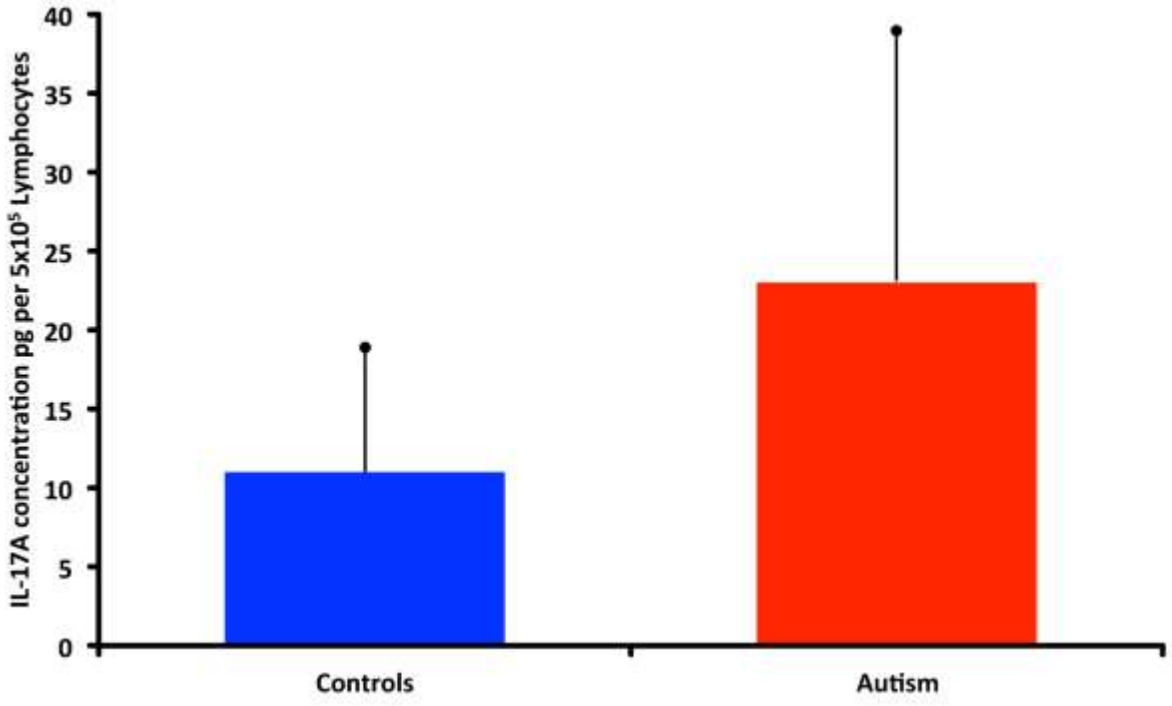

Figure 11. Comparison of IL-17A production between controls and a subgroup of children with ASD suffering from inflammation and autoimmunity, particularly, neuroautoimmunity. Results from 113 healthy controls and 200 children with autism showed that a subgroup of 28% of ASD children had a significant elevation in IL-17A production.

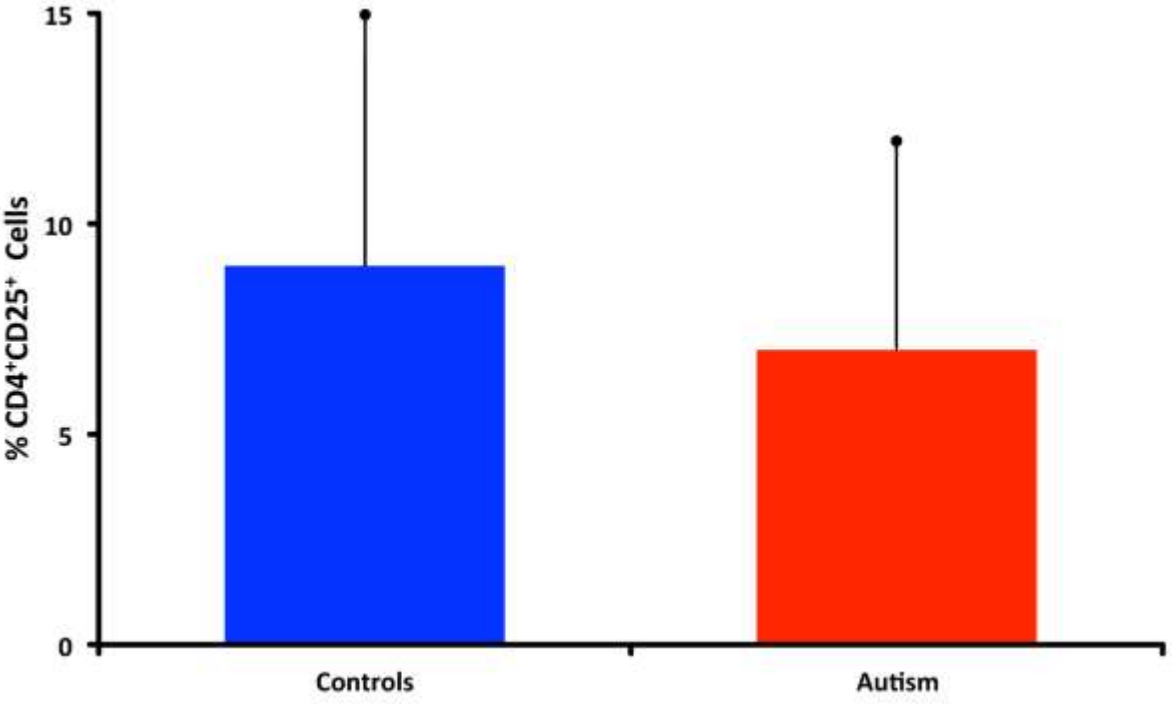

Figure 12. Low percentage of $CD4^+CD25^+$ regulatory T cells from 113 healthy controls and 200 children with ASD.

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6. Discussion

In our review article in this same issue, we established that the possible root cause of ASD was the combination of genetic variation and environmental triggers. We also hypothesized that the effect of environmental factors on early life immunology may result down the line in oral tolerance failure, change in the gut microbiome, intestinal hyperpermeability, and possible autoimmunities.[64] Here we have illustrated the use of biomarkers to identify environmental insults, barrier breakdown and immune dysregulation as seen in ASD. These test results provide possible therapeutic opportunities for the treatment of children afflicted with ASD.

Understanding the microbiome is essential. In a very interesting review article by Blaser in *Nature Reviews: Immunology* entitled "The theory of disappearing microbiota and the epidemics of chronic disease," the author elegantly discusses how the interactions of the early life microbiome with the host sets the immunological reactions for the remainder of the individual's lifespan.[34] He postulates that, unfortunately, elements and circumstances of modern living, especially those encountered during an individual's earliest formative stage, have led to the extinction or disappearance of beneficial ancestral microorganisms, resulting in the maturing immune system becomes less tolerogenic and more reactogenic. This reactogenic immune system is what is fueling the current epidemics of autoimmune and inflammatory diseases,[34] including the rising incidences of ASD.[34]

Because diet is an important modulator of gut microbiota[74] and the gut microbiomes of people with ASD is different from those of healthy controls,[41,75] we started our search for useful biomarkers by looking at immune reactivity to dietary proteins. A variety of assessments for dietary protein reactivity has been utilized by practitioners. The two key concepts of assessing dietary proteins are: 1) the food panel must be reflective of what the patient consumes and 2) the food antigens used in the performance of the test must be pure. Without these standards, food immune reactivity will result in false negatives or false positives.

Common immunogenic foods, such as wheat and dairy, are made up of a variety of proteins. Thus, in testing immune reactivity to these foods, the best practice would be to assess multiple proteins from wheat and dairy. In an earlier study,[76] we did such an assessment with the blood samples of 400 healthy subjects and concluded that a subgroup of individuals, due to a breakdown in immunological tolerance, may react and produce significant levels of antibodies against wheat and milk antigens. These antibodies also cross-referenced with neural antibodies.[76]

Furthermore, extending these findings to autism, Crohn's and Celiac disease (CD), in a recent study[71] we measured antibodies against various gluten and non-gluten wheat proteins. Compared to controls in all three conditions, the strongest reaction was against CXCR3-binding gliadin peptide, followed by a wheat protein mixture, then α-gliadin 33-mer peptide. We determined that a sample that reacted strongly against non-gluten proteins also reacted strongly against gluten proteins. We also found that IgA antibodies against CXCR3-binding

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gliadin peptide were strongly reactive in a subgroup of 33% in the autism group, 42% in the Crohn's group, and all tested samples with CD.

This elevation in antibodies against dietary components is not unique to wheat and dairy in ASD. Other food antigens could be involved.[14] This was previously reviewed by the author.[77] Due to low activity of digestive enzymes in ASD patients, food antigens from different sources may survive as intact proteins or peptides, and the immune reaction against them may result in antibody production against the food proteins or peptides. If there is antigenic similarity between the food protein or peptide and human tissue structures, the food antibodies may cross-react with tissue antigens. The end result could be autoimmune reactivity followed by autoimmune disease.[77-80]

Chemicals such as mercury, aluminum, and formaldehyde are not only part of our environment but are also used as vaccine additives or adjuvants. Because of the mother's own exposure to these chemicals in her environment, children with ASD have thus been exposed to such toxins through their mother's blood both while in the womb and through breastfeeding, as well as through repeated vaccinations. A certain percentage of these haptenic chemicals form alliances with human tissue antigens, including albumin.

Because of this neo-antigen formation, antibodies are produced against mercury, aluminum, formaldehyde, and other chemicals bound to tissue and the released cellular proteins.[15] On a daily basis, more than 200 mg of aluminum, through alum-treated drinking water and via food products, is ingested by humans. In addition, many vaccines contain aluminum. Thus, a very high percentage of aluminum is available for uptake by various organs and tissue proteins in the body. The detection of antibodies against aluminum adducts in healthy controls and ASD patients is an indication, not only of exposure to aluminum, but also of neo-antigen formation with aluminum binding to tissue macromolecules. This sets the stage for autoimmune reactivity commonly detected in ASDs.

Similarly, BPA and its metabolites can bind to human tissue, especially to protein disulfide isomerase (PDI), its target enzyme in astrocytes. Because PDI is involved in the prevention of protein misfolding, the binding of BPA to PDI may result in the misfolding of neural cell antigens such as α-synuclein, and hence may induce neuroautoimmunity and neurodegeneration.[81]

Food colorants are another group of chemicals that bind covalently to human tissue to form neo-antigens, causing the production of antibodies against both the colorants and the human tissue.[82] Colorant binding to tissue is the first step in the induction of autoimmune and neuroautoimmune reactivities in ASD.[82]

Glyphosate, which has been marketed commercially as Roundup®, is a non-selective herbicide that is used worldwide. Because of its chemical structure (*N*-(phosphonomethyl)glycine), especially its component carboxylic group, it can form neo-antigens with human tissue.[70] Based on this mechanism of action, the pathologies to which glyphosate could possibly contribute

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through its known biosemiotic effects include inflammatory bowel disease, obesity, depression, ADHD, autism, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, cancer, cachexia, infertility, and developmental malformations. Glyphosate works synergistically with other factors, such as insufficient sun exposure, dietary deficiencies in critical nutrients such as sulfur and zinc, and synergistic exposure to other xenobiotics whose detoxification in impaired by glyphosate.[29-37,83,84]

A few scientists believe that glyphosate may be the most significant environmental toxin contributing to autism. While it is pervasive in our food supply, the fact that it is deemed by most regulators to be non-toxic makes it especially insidious.[31,37,83,84] The binding of glyphosate to body proteins as shown by Yue *et al*.,[70] the production of antibodies against it in healthy controls, and the much higher levels seen in patients with ASD as shown in the present study opens a new chapter about the role of glyphosate in the field of environmental toxicology, and possibly autoimmunity. The key pathological biological effects of glyphosate and other chemicals is disruption of the gut bacteria, impairment of sulfate transport, and interference with cytochrome P450 enzyme activity, which can easily explain the features that are characteristic of autism.[31,51] Previous studies have shown gut dysbiosis in patients with ASD[42,75,81] and the manipulation of the enteric microbiome being use to improve autism symptoms.[42]

Lipopolysaccharides (LPS) and bacterial cytotoxins are examples of bacterial toxins produced by a variety of gram-negative bacteria such as *E. coli, Shigella, Salmonella, Campylobacter jejuni* and *Klebsiella.* These bacterial toxins have the capacity to break down tight junction proteins such a occludin/zonulin and vinculin.[43,44] Once these inflammatory agents reach the bloodstream, they target tissues, including those of the blood-brain barrier (BBB), leading to its opening and allowing penetration into the brain, where they can trigger seizures, depression, and autoimmunity.[85,86] Based on this mechanism of action, we measured antibodies against bacterial toxins, tight junction proteins, BBBs, and neuron-specific antigens, and found them to ne highly elevated in patients with ASD compared to controls. These antibodies may play a role in the induction of the neuroinflammation seen in ASD.[39-51]

Specific food aquaporins, notably corn, spinach, soy, and tomato share homology with the human aquaporin expressed in astrocytic foot processes.[87] Circulating antibodies to food aquaporins may therefore cross-react with the aquaporin supporting the BBB, resulting in a broken BBB. Upon breach of the BBB and subsequent microglia activation, myelin oligodendrocyte glycoprotein (MOG) is released by oligodendrocytes while myelin basic protein (MBP), cerebellar peptide and synapsin are produced by neurons.[88,89] Depending on which region of the brain is targeted by environmental insults, antibodies are produced against these neurological tissues.

In ASD, in addition to humoral immunity, cell-mediated immunity also plays a role. Cell-mediated immune responses include macrophages, T cells, B cells, helper cells, suppressor cells, natural killer (NK) cells,

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memory lymphocytes, cytokine production, and regulatory T cells (Tregs). Humoral immune responses include secretory IgA (SIgA), immunoglobulin G, A, M, E, and IgG subclasses. Together these immune responses function as a harmonious symphonic orchestra. When disharmony occurs or the tempo loses the beat, the immune dysfunction, due to elevated inflammatory cytokines and down-regulation of regulatory cytokines, may contribute to ASD.[66,70] We found that in the ASD group these major inflammatory cytokines were elevated: IL-1β, TNF-α, IL-6, and IL-17 (Figures 10 and 11). Conversely, we found that in the ASD group the production of the two major cytokines IL-10 and TGF-β were much lower than in controls ($p < 0.0001$).

In another earlier study,[90] serum levels of IL-17A were measured in 45 children with autism and 40 matched healthy controls. Overall, children with autism showed significantly higher serum IL-17A levels. Raised serum levels of IL-17A were found in 68% of patients with severe autism compared to children with mild or moderate autism, in which about 18% exhibited IL-17A elevation.[90]

Due to its known role in neuroinflammatory disorders,[91-93] the increased production of IL-17A in a subgroup of children with ASD may be one of the factors associated with Th17 penetration of the BBB and the induction of brain-specific antibody production in some children with ASD, as reported in these studies.

The term "developmental immunotoxicity" has been coined to describe permanent modifications to the immune function that take place early in life, leading to later development of allergies, asthma, and autoimmune diseases. Many authors have argued that prenatal and/or early life exposure to environmental toxins can lead to a phenotype that includes a hyperinflammatory response and disruption of cytokine networks, and they propose that an increased exposure to environmental toxins early in life may contribute to the increased incidence of these conditions observed today.[94-96]

7. Conclusion

Autism spectrum disorders can be challenging to assess and manage. Since ASD can have both environmental and genetic roots, the multiplicity of environmental triggers and genetic variations and their combinations leads to the diverse presentations of the condition that can make it difficult to identify, diagnose and treat. It is necessary to thoroughly understand the connections between these triggers, genes, failures of the immune system, dysfunctions in the gut and brain barriers, and autoimmune diseases so that healthcare practitioners can better assess, recover and manage their ASD patients. In this article we have shown how the very food we eat, the chemicals that are all around us in every aspect of our lives, and common infectious diseases can promote breakdown of the immune barriers, the failure of immune tolerance and dysfunction of the immune system. We have shown how vital the intestinal and blood-brain barriers are in protecting the body and the brain from the assault of pathogenic invaders, but when a breach does occur for any number of reasons, the body's

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own autoimmune mechanisms can turn on it and attack its own body including neural tissues.

Because there are so many variables involved in the environmental factors that may act as triggers for the disease, so far there has been no consensus on the definitive causes of ASD, and no one has come up with a single convincing test for it.[8,9] What researchers must understand is that the pathogenesis of ASD comes, not just from one cause, but rather, is the result of a combination of inflammatory cytokines, molecular mimicry between environmental immunogens such as dietary proteins, or bacterial toxins, and tissue proteins, and the binding of environmental factors such as food proteins or chemicals to human tissues, any of which could lead to neuroautoimmunity. The multi-faceted characteristics of the disorder means an array of assessments must be utilized in order to identify the environmental triggers involved, the type and severity of barrier damage and pinpoint which neurological tissue is targeted. There is no "one-size fits all" option for assessing spectrum disorders, which is seen in the discrepancy of study outcomes.[8,9,57-64] By utilizing the system of tests outlined in this article, the healthcare practitioner can individualize the treatment, management and, hopefully, recovery of children with ASD. The blood-based humoral markers in ASD should be selected from environmental factors such as food, gut bacteria, infectious agents, xenobiotics, and antibodies against various human tissue antigens. Finally, we should not forget the role of cellular immune function assessment in diagnosing and treating ASD. This includes lymphocyte subpopulation with emphasis on regulatory T cells, T-cell function, B-cell function, NK cell cytotoxic activity, and inflammatory versus anti-inflammatory or regulatory cytokines. The development of biomarkers such as those used in this study is essential for improving the diagnosis, management, and development of new therapies for ASD.

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