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Biomarker-Guided Interventions of Clinically Relevant Conditions Associated with Autism Spectrum Disorders and Attention Deficit Hyperactivity Disorder

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Abstract

Autism spectrum disorders (ASD) and attention-deficit hyperactivity disorder (ADHD) are common and complex neurodevelopmental conditions. Diagnostic criteria for these conditions have traditionally relied solely on behavioral criteria without consideration for potential biomedical underpinnings. Newer evidence, however, reveals that ASDs are associated with: oxidative stress; decreased methylation capacity; limited production of glutathione; mitochondrial dysfunction; intestinal dysbiosis; increased toxic metal burden; immune dysregulation, characterized by a unique inflammatory bowel disease and immune activation of neuroglial cells; and ongoing brain hypoperfusion. Many of these same problems are common features in children with ADHD. These medical conditions, whether co-morbidities or etiopathogenic, would be expected to have synergistically negative effects on the development, cognition, focus, and attention of affected children. It is likely these biological abnormalities contribute significantly to the behavioral symptoms intrinsic in these diagnoses. However, treatment for these underlying medical disorders is clinically justified, even if no clear immediate behavioral improvements are observed. This article reviews the medical literature and discusses the authors' clinical experience using various biomarkers for measuring oxidative stress, methylation capacity and transsulfuration, immune function, gastrointestinal problems, and toxic metal burden. These biomarkers provide useful guides for selection, efficacy, and sufficiency of biomedical interventions. The use of these biomarkers is of great importance in young children with ADHD or individuals of any age with ASD, because typically they cannot adequately communicate regarding their symptoms. (*Altern Med Rev* 2010;15(1):15-32)

Background

Autism (autistic disorder), Asperger syndrome, and pervasive developmental disorder (not otherwise specified) comprise a heterogeneous spectrum of neurodevelopmental disorders collectively termed autism spectrum disorders (ASD). They are behaviorally defined and characterized by restrictive and repetitive behaviors along with impairments in communication and social interaction. The number of children diagnosed with ASD has substantially increased over the last decade[1-3] and ASD currently affects an estimated one out of 91 individuals in the United States.[4] However, since ASD occurs four times as frequently in males than females,[5] reporting the prevalence of ASD in all children significantly underestimates the number of affected males. A reasonable extraction of the overall data when applied to the male population finds that one in 58 are likely affected with ASD,[4] and the prevalence of affected males approaches two percent of the general population in additional studies.[6,7] ASD is traditionally considered a "static" encephalopathic disorder[8] without any known cure and few proven effective biomedical interventions. Furthermore, attention-deficit hyperactivity disorder (ADHD), which affects 4-12 percent of school age children,[9] is behaviorally characterized by features of inattention, hyperactivity, and impulsivity.[10] While ADHD and ASD present complex medical problems for physicians, interventional strategies may be streamlined for many children as a result of advances in biomarker research. Given the large number of affected children and the continued increase in prevalence of both disorders, a simplified treatment approach is needed for implementation by primary care providers.

Recent evidence reveals that many children with ASD have multiple medical problems, including increased oxidative stress,[11-13] decreased methylation capacity with limited transsulfuration,[12,14] mitochondrial dysfunction,[15,16] increased toxic metal burden,[17-21] intestinal dysbiosis skewed toward an overgrowth of *Clostridia* species,[22-24] immune dysregulation with a unique inflammatory bowel disease and immune activation of glial cells in the brain,[25-28] combined with central nervous system (CNS) hypoperfusion or abnormal regulation of blood supply to the brain.[29,30] Furthermore, some of these medical problems, including oxidative stress,[31-33] metal toxicity,[34,35] decreased methylation,[36] mitochondrial dysfunction,[37] and cerebral hypoperfusion[38] have also been described in children with ADHD (Table 1). A review of approximately 4,000 records of children evaluated at our centers with predominately ASD diagnoses affirms the frequent co-occurrence of these underlying biological problems. Certainly these factors adversely impact neurodevelopment, immune function, and gastrointestinal (GI) health. The difficulties of evaluating the synergistically negative effects of these abnormalities in the pediatric population will likely preclude controlled interventional studies. Given the broad array of pediatric sub-specialties typically involved in these disorders (e.g., neurology, psychiatry, gastroenterology, immunology, and toxicology), it is a daunting task for a single medical provider to align the skills and expertise necessary to integrate appropriate care. Importantly, the presence of behavioral symptoms consistent with autism or ADHD does not necessarily preclude the recovery from or diminishing of these symptoms through the treatment of underlying pathophysiologies. In fact, recovery from autism, although not widely published, is commonly observed. Consistent with the authors' observations of recovery assisted by biomedical interventions, O'Hara and Szakacs recently published the recovery from autism in one child.[39] Other investigators have also reported recovery of 38 children with autism primarily through Applied Behavioral Analysis (ABA) Therapy.[40] In the ABA literature, normalization of IQ and behavior is consistently reported,[41-43] which implies reversibility of the underlying condition in at least some children.

Despite numerous challenges, children with these medical disorders deserve the hope of a better quality of life and the possible recovery from the core features of their disorders. Fortunately, the progress made in defining the underlying processes of these conditions has led to numerous published studies that define clinically useful and commercially available biomarkers for both ASD and ADHD. Based on this body of medical literature, as well as the authors' extensive clinical experience over the past 12 years, unless the underlying major biological disruptions are addressed, they will perpetuate autistic and ADHD symptoms, adversely impact the child's development, and prevent potential improvements in symptoms and overall functioning.

Researchers have examined the use of biomarkers in children with ASD for over 20 years.[44] Chakravarty defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes or pharmacological responses to a therapeutic intervention."[45] Biomarkers do not need to be exclusive to a particular disorder. For example, oxidative stress is reported as a common feature of vascular diseases[46] as well as many CNS disorders, including ASD, schizophrenia,

Table 1. Biomedical Problems Described in ASD and ADHD

BIOMEDICAL PROBLEM	ASD	ADHD
Oxidative stress	Yes	Yes
Decreased methylation and transsulfuration	Yes	Yes
Mitochondrial dysfunction	Yes	Yes
Metal toxicity	Yes	Yes
Intestinal dysbiosis	Yes	No
Immune dysregulation / inflammation	Yes	No
Cerebral hypoperfusion	Yes	Yes

Alzheimer's disease, HIV-dementia, and parkinsonism.[47,48] Oxidative stress therefore represents a common etiopathological factor of diverse clinical conditions, but cannot be used as a specific diagnostic requirement of any exclusive disorder. It is medically reasonable to assume that the relief of oxidative stress would be associated with diminution of some features of these disorders, or at least

Table 2. Basic Biomarkers and Clinical Significance

Basic Biomarker	Clinical Significance
Complete blood count	Anemia, abnormal white count, platelet count
Comprehensive metabolic	Electrolyte, liver, or renal abnormalities
Magnesium	Deficiency associated with hyperactivity
Zinc	Deficiency associated with inattention
Other minerals	Low chromium associated with pica; low lithium associated with irritability
Iron	Deficiency associated with insomnia, lower IQ, attention problems
Hypothyroidism	Deficiency associated with developmental delay and inattention
Cholesterol	Deficiency associated with irritability
Testosterone	Increase associated with aggression

prevent or slow their progression. The biomarkers described in this article are not exhaustive or all inclusive, but are intended to target the core biomedical issues frequently observed in children with ASD or ADHD. Utilizing biomarkers is of greater importance in individuals with ASD, because they typically cannot adequately communicate their symptoms. As with any medical diagnostic evaluation, a clinician must rely on the history, physical examination, and relevant biomarkers for proper diagnosis and treatment.

Basic Biomarkers

Several abnormalities described in children with ADHD and ASD that can be screened with simple laboratory tests are summarized in Table 2.

▸ CBC: A complete blood count (CBC) with differential can be performed. Abnormalities described in some children with ASD include a high blood monocyte count[49] and abnormal lymphocyte function.[50-53] The CBC can also provide insights into allergies, anemia, and platelet counts. Platelet elevation, a nonspecific marker of immune activation, were observed in

ASD and were responsive to biomedical intervention.[162]

▸ CMP: A comprehensive metabolic panel (CMP) that includes liver and kidney testing is helpful. High albumin has been described in some children with ASD.[54] Elevations in transaminases can be associated with mitochondrial disorders and, along with other markers, may support the need for skin or muscle biopsy for a more definitive diagnosis.[55,56] Determining renal and hepatic function prior to intervention with medications represents a reasonable clinical protocol.

▸ Magnesium: Magnesium (Mg) deficiency, which can be measured by any standard laboratory, occurs in up to 95 percent of children with ADHD.[57] In a six-month, controlled study of 75 children with ADHD and magnesium deficiency (documented by low serum and red blood cell [RBC] magnesium) who all received standard pharmacological treatments for ADHD, a significant decrease in hyperactivity was observed with the addition of oral magnesium (200 mg/day) in 50 children compared to the 25 children who did not receive magnesium ($p < 0.05$).[58] In a six-month, controlled study of 33 children with ASD, the use of vitamin B_6 (0.6 mg/kg/day) and magnesium (6 mg/kg/day) led to a significant reduction of autistic symptoms in 70 percent of the children ($p < 0.0001$), including improvements in social interaction, communication, and stereotypies; no adverse effects were observed. When the B_6/Mg treatment was stopped the undesired behavior returned within several weeks.[59]

▸ Zinc: Zinc can be measured by any standard laboratory. In one study of 48 children with ADHD and 45 typically developing children, mean serum zinc levels were significantly lower in the ADHD group ($p < 0.001$).[60] Other investigators studied a group of 48 children with ADHD and observed that lower serum zinc levels correlated with parent and teacher rankings of inattention ($p = 0.004$ for both).[61] In a controlled study of 45 autistic children compared to 41 typically developing children, plasma and RBC zinc levels were significantly lower in the autism group ($p < 0.05$).[62]

▸ Other minerals: One study reported that children with ASD and pica had lower hair chromium.[63] Low hair iodine and lithium levels have also been described in some children with ASD.[63] A study of 20 children with autism and 15

typically developing children reported significantly lower RBC selenium (p<0.0006) in the autism group.[64] A reasonable method to determine mineral content is to assess packed red blood cell (PRBC) element concentrations, a technique that has been evaluated in the pediatric population.[65]

- Iron: Iron deficiency appears to be relatively common in ADHD;[66] serum ferritin is low in many children with ADHD compared to typically developing children.[67,68] Iron deficiency characterized by low serum ferritin is also observed in many children with ASD.[69,70] In a randomized, double-blind, placebo-controlled study of 23 ADHD children with serum ferritin levels less than 30 ng/mL, supplementation with ferrous sulfate (80 mg/day) over a 12-week period was well-tolerated and significantly improved ADHD symptoms (p<0.008) compared to no improvements in the placebo group.[71] In an eight-week, open-label study of 33 children with ASD, supplementation with iron (6 mg/kg/day) significantly improved sleep and increased mean serum ferritin levels. The investigators suggested that children with ASD should be routinely screened for iron deficiency and recommended obtaining serum ferritin and iron levels.[72]

- Hypothyroidism: Hypothyroidism has been described in some children with ASD[73] and ADHD;[74] therefore, screening for hypothyroidism with a blood test for thyroid-stimulating hormone is recommended. Normal ranges for children vary among laboratories. It is not unusual to see two standard deviations signify a 10-fold difference in TSH levels. TSH levels at the mean or lower are considered optimal by these authors. Elevated TSH may be a reflection of iodine deficiency, an easily corrected nutritional problem.

- Cholesterol: A subset of children with ASD have abnormally low cholesterol levels, with one study demonstrating that 19 percent of children had a cholesterol level below 100 mg/dL.[75] Cholesterol levels below 145 mg/dL have been associated with a three-fold increased risk of aggression and suspension from school in typically developing children.[76]

- Testosterone: A small percentage of children with ASD may have elevated testosterone levels.[77] Elevated fetal testosterone levels also appear to be associated with a higher likelihood of developing ASD.[78] Thus, measuring levels of serum testosterone and related androgens may be indicated. In the authors' clinical experience, the typical features of precocious puberty may not be present in all hyperandrogenic states. If indicated by height percentiles, a wrist radiograph for bone age may also be helpful. A child with a bone age that is advanced more than two standard deviations, when combined with elevated androgens, should be considered for a complete precocious puberty evaluation.

Oxidative Stress Biomarkers

Oxidative stress is a common finding in many children with ASD[12,13,47] and ADHD.[31-33] Glutathione is the primary intracellular antioxidant and has been shown to limit mercury-induced neurotoxicity.[79] Impaired glutathione production contributes to oxidative stress, which may delay the clearance of heavy metals and certain xenobiotics.[80] In two prospective studies, over 50 percent of children with ASD had significantly lower plasma levels of glutathione and cysteine (p<0.001 for both) compared to typically developing children.[12,14] James et al hypothesized that because of these findings, "autistic children would be expected to have difficulty resisting infection, resolving inflammation, and detoxifying environmental contaminants."[12] The following biomarkers, summarized in Table 3, can be measured to assess the level of oxidative stress.

- Reduced glutathione (GSH) and oxidized glutathione (GSSG):[12] An Internet search of laboratory providers for this special testing found several commercially available companies capable of measuring these valuable markers. Measuring total glutathione along with GSSG and/or GSH will help determine the patient's oxidation status.

- Levels of major antioxidant proteins in the serum (standard blood tests): Transferrin (an iron-binding protein) and ceruloplasmin (a copper-binding protein) are antioxidant proteins significantly decreased in children with ASD compared to typically developing children.[13,81] One study reported that lower levels of these proteins were associated with regression and loss of previously acquired language skills in children with ASD.[81] Results of such testing should be viewed with caution, however, since a variety of conditions influence the levels of either protein, making interpretation challenging.

◗ Blood ammonia and lactate (lactic acid): Ammonia is derived from the deamination of the amine group of amino acids by gut bacteria or the liver. The process of detoxifying ammonia via the urea cycle is metabolically expensive and expends three valuable, high-energy ATP molecules for every ammonia molecule processed. Hyperammonemia is more toxic for children than adults and can lead to permanent CNS damage.[82]

Table 3. Oxidative Stress Biomarkers

Biomarker	Clinical Significance
Reduced and oxidized glutathione	Low levels associated with impaired detoxification and increased oxidative stress
Antioxidant proteins: transferrin and ceruloplasmin	Low levels associated with regression in children with autism
Ammonia and lactic acid	High levels are nonspecific markers of mitochondrial dysfunction
Carnitine profile	Low levels associated with mitochondrial dysfunction
Urinary 8-hydroxyguanine	Increased levels associated with mitochondrial dysfunction and oxidative stress
Urinary isoprostane	Increased levels consistent with fatty acid oxidation
Vitamin D	Low levels associated with lowered glutathione levels and increased oxidative stress

◗ Lactate is a by-product of the anaerobic metabolism of glucose. Typically, clinicians look for serum lactate levels greater than 2.5 mM/L for support of mitochondrial disease,[83] although lactate levels can be normal in some mitochondrial diseases.[84] Lactate can be elevated in a variety of disorders other than ASD, but levels above 2 mM/L support mitochondrial dysfunction when proper sampling techniques are followed. When possible, lactate and ammonia levels should be drawn without a tourniquet after the venipuncture or IV is started. Ideally, the child should be calm or sedated during the process and may require premedication to obtain accurate results. Increased lactate levels may require confirmation with a separate blood draw. Elevation in either ammonia or lactate likely reflects a state of mitochondrial hypofunctioning in ASD[15,85,86] and are standard tests at all hospitals. The blood used for ammonia and lactate testing requires immediate icing once placed in the specimen tubes. As a precautionary note when interpreting the meaning of elevated serum lactate, the authors have observed elevations after high-dose probiotic supplementation with *Lactobacillus* species, secondary to the bacterial metabolic contribution to blood lactate levels.

◗ Serum carnitine profile: Carnitine levels are often lower in children with ASD[15] and may reflect mitochondrial dysfunction and nutritional deficiencies. This test should be routine for any child with hypotonia or other signs and symptoms of mitochondrial dysfunction.

◗ Urinary 8-hydroxyguanine (8-OHG): This is a marker of RNA oxidation in the mitochondria and cell cytoplasm and is an easily obtained urinary marker useful for evaluating intracellular oxidative stress.[87] Although the DNA marker of oxidative stress (8-hydroxy-2-deoxyguanosine, 8-OHdG) is not elevated in most cases of ASD,[88] it is elevated in some children with ADHD.[33] As far as the authors are aware, 8-OHG testing is currently commercially available only from Laboratoire Philippe Auguste (Paris, France), although several U.S. laboratories can measure DNA oxidation.

◗ Urinary isoprostane: This is a marker of fatty acid oxidation that reflects cell membrane (extracellular) oxidative stress and is elevated in many children with ASD when compared to controls.[88] This test is available from Laboratoire Philippe Auguste (Paris, France).

◗ Vitamin D: Deficiency of vitamin D is an emerging concern among many practitioners. It is interesting to note that vitamin D deficiency and autism share the common qualities of enlarged brain size and ventricles.[89,90] The authors have also observed increased rates of autism in some darker-skinned populations and insufficient vitamin D may be partly responsible.[91] Vitamin D status is important to consider because of its role in reducing oxidative

stress through both GSH production and as an antioxidant itself.[92-94] Vitamin D panels are available from many commercial laboratories. When evaluating a potential deficiency state, a decrease of the 25-hydroxycholecalciferol form is diagnostic of inadequate dietary intake.

Methylation Capacity and Transsulfuration Biomarkers

Methylation and transsulfuration pathways represent core areas of metabolic activity. These connected and interdependent pathways generate required methyl-donors via the conversion of methionine to S-adenosylmethionine (SAMe), which in turn donates its methyl group to catecholamine neurotransmitters, cell membranes, DNA, and other body chemicals or structures. The end product is homocysteine, which is merely demethylated methionine. Excess homocysteine is required to generate cysteine, the rate-limiting step for the production of the vital and dominate intracellular antioxidant glutathione. An oxidized intracellular condition would inhibit the methionine cycle, making the reducing capacity of glutathione critical to its own production. It is well accepted that neurons are extremely sensitive to oxidation, making GSH essential for neuronal survival.[95] James et al documented methylation and transsulfuration disruption in a majority of children with ASD.[12,14,96] Deficiency, as measured by decreased levels of fasting plasma cysteine or its dimer (cystine), may predict improvement with methylcobalamin injections and/or folinic acid.[14,96] The use of 5-methyltetrahydrofolate (5-MTHF) may also be useful. Logically, deficits in this pathway could be the result of nutritional deficiencies of methionine (an essential amino acid), folate, and/or vitamin B12 along with other vitamin cofactors. As mentioned, deficient methylation-transsulfuration could also be the result of increased oxidative stress. The following biomarkers, summarized in Table 4, can be checked to assess methylation and transsulfuration pathways.

- Fasting plasma cysteine or cystine: Cysteine is the sulfur-containing amino acid that acts as the rate-limiting step in the production of GSH, the key intracellular defense against oxidative stress. Cysteine and GSH are also involved in defending against heavy metal and xenobiotic toxicity.
- Fasting plasma methionine: Methionine, an essential amino acid, is the main methyl donor via the intermediary SAMe.

Table 4. Biomarkers of Methylation and Transsulfuration

Biomarker	Clinical Significance
Cysteine or cystine	Low levels associated with impaired glutathione production
Methionine	Low levels associated with impaired glutathione production
Sulfate	Low levels associated with impaired detoxification

- Plasma sulfate: Sulfate is often low in children with ASD.[97] Several studies report that the conjugation of sulfate to acetaminophen (an indicator of deficient phase 2 hepatic sulfation-detoxification) is impaired in children with ASD.[98-100] Deficient sulfation in turn may impair the detoxification of many chemicals and metabolites, such as phenolic xenobiotics that could adversely impact brain function.[101]

Immune Biomarkers

It is difficult to obtain a direct measurement of brain inflammation; even cerebral spinal fluid (CSF) studies offer inconsistent findings. Given the literature demonstrating the presence of both cerebral oxidation and inflammation in ASD, a pattern of up-regulation of cellular immunity combined with other features of immune dysregulation can help form a clinical picture. The following biomarkers, summarized in Table 5, may reflect this immune dysregulation.

- Serum autoantibodies to brain endovasculature:[102,103] This test is performed exclusively at the Neuromuscular Laboratory at Washington University in St. Louis, MO. Details related to specimen handling and requirements are available at their website. In the authors' practice, the presence of either IgG or IgM antibodies to brain endovasculature is common (exceeding 50% of ASD) and predicts speech delay or regression. It is consistent with and probably (although unconfirmed) a marker for autoimmune vasculitis of the brain, as depicted in the brain immune studies of Vargas et al.[28]
- Neopterin and biopterin: These biomarkers are often elevated in the urine and monocytes of

Table 5. Biomarkers of Immune Dysregulation

Biomarker	Clinical Significance
Serum autoantibodies to brain endovasculature	Associated with speech delay and speech regression in children with ASD
Neopterin and biopterin	Measures of cell-mediated immune activation
Immunoglobulin subsets	Markers of immunodeficiency; low levels also correlate with core autistic symptoms
Vaccine titers	Lack of antibody production after immunization can be a marker of immunodeficiency
Antinuclear antibodies	Reflect autoimmunity
Urinary N-methylhistamine	High levels consistent with inflammatory bowel disease
Tumor necrosis factor-alpha	Generalized marker of inflammation
PANDAS biomarkers	Confirm previous exposure to GABHS in children without obvious strep exposure history

children with ASD compared to typically developing children.[49,104,105] Neopterin predicts the degree of cell-mediated immune activation and biopterin measures the immune system's attempt to compensate for oxidative stress induced by immune activation. Laboratoire Philippe Auguste (Paris, France) is the only commercial laboratory the authors are aware of with pediatric controls for urinary studies. A significant correlation between elevated urinary neopterin and favorable clinical responses to immune interventions has been observed by the authors.

➧ Immunoglobulin subsets: IgG (subclasses 1-4), IgM, IgA, and IgE. High IgG, IgG2, and IgG4 levels have been described in a small subset of children with ASD.[54] While this does occur, the authors' experience parallels that of Gupta et al, with deficiencies of IgG subclasses, IgA, and IgM along with specific cellular immune deficiencies being more common than increased levels of globulins.[106] One recent controlled study

reported plasma immunoglobulin levels in 116 children with autism were significantly lower than those of 96 typically developing children (p<0.001), and that children with the lowest levels had the highest autism severity as rated on the Aberrant Behavior Checklist (p<0.0001).[107] Oleske observed that in a subset of children with ASD, immune deficiency predicted a favorable clinical response to intravenous immunoglobulin (IVIG) therapy.[108] IgE is elevated in some children with ASD[109,110] and IgA is low in a subset of children.[109,111] Extreme IgA deficiency is rare, but is important to exclude prior to starting IVIG therapy, as the treatment of an extremely IgA-deficient child requires special product selection to prevent anaphylaxis.[112]

➧ Vaccine and specific antibody titer testing: This test is useful in defining specific antibody deficiencies to critical antigens such as streptococcal pneumonia or vaccine-related antigens. Deficiency of specific antibody responses in the presence of recurrent infections may be an indication for IVIG therapy.

➧ Antinuclear antibodies (ANA): ANA are known to reflect autoimmunity and are elevated in a subset of children with ASD.[103]

➧ Urinary N-methylhistamine testing: This test may be useful in some cases of ASD and is a biomarker of significant inflammatory bowel disease.[113] N-methylhistamine is also elevated in asthma.

➧ Tumor necrosis factor-alpha (TNF-α): Chez et al observed a markedly increased ratio of CSF to serum TNF-α in children with ASD.[114] This is an intriguing observation, which could represent an ideal way to assess the inflammatory state of the CNS. Although it is invasive, it is a test that deserves more attention despite the expected poor parental acceptance of CSF testing.

➧ Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection (PANDAS): Giedd et al first reported PANDAS in 1996.[115] The disorder is characterized by the acute onset of obsessive compulsive disorder (OCD) and often tics following a Group A beta-hemolytic streptococci (GABHS) infection. The National Institute of Mental Health (NIMH) group studying this found streptococci- (strep) induced autoantibodies to basal ganglion. This is much the same as the immune system

forming cross-reactive antibodies between GABHS and heart valves in rheumatic heart disease. Both OCD and tic exacerbations are often observed in the ASD population, but cultures are often negative or there is no clear antecedent strep infection. This makes the diagnosis of PANDAS challenging since, apart from the clinical picture of a positive throat culture preceding the onset of new OCD and tic behaviors, there is no clinically reliable diagnostic testing available. The basal ganglion autoantibody tests are not commercially available and traditional antibody testing for strep (anti-streptolysin-O and anti-DNase B) can be deceptive since many healthy children carry high titers for a long time after infection or during strep outbreaks at school (personal communication from Sue Swedo, MD, NIMH). Although treatment for this condition is controversial, prolonged courses of antibiotics have been proposed[116] and in the authors' experience may be helpful in select ASD-PANDAS cases. The group at NIMH is also investigating novel treatments, including immunomodulatory therapies such as therapeutic plasma exchange (TPE) and IVIG, with some benefits noted.[117] The authors, although not experienced with TPE, have found monthly infusions of IVIG are needed for extended periods of time in order to see improvements when ASD and PANDAS-like symptoms occur together.

Gastrointestinal Biomarkers

Gastrointestinal inflammation has been described in many children with ASD.[27,118,119] Other GI problems (reflux, constipation, food sensitivities, and abnormal flora) are also common. For example, Horvath et al reported significant GI symptoms in a study of 36 autistic children referred to a gastroenterologist, including reflux esophagitis (69%), chronic gastritis (42%), chronic duodenitis (67%), and low intestinal carbohydrate digestive enzyme activity (58%).[120] Another study of 50 children with ASD and 50 typically developing children reported that 70 percent of the ASD group had a history of GI problems compared to 28 percent of the control group (p<0.001).[121] Common GI problems found in one study of 112 autistic children included diarrhea (28%), gaseousness (60%), bloating (38%), abdominal pain (38%), and fecal impaction (19%). Importantly, 80 percent of the children with autism had at least one GI-related

problem and these problems were significantly more common compared to 44 typically developing siblings.[122] Several studies also report dysbiosis in children with ASD, including significant overgrowth of *Clostridia* species[22-24] and yeast (*Candida albicans*)[123] in the GI tract compared to typically developing children. The following biomarkers, summarized in Table 6, can be obtained to assess these problems.

◗ Fecal testing: Calprotectin,[124,125] eosinophil X,[126] and S100A12[127] are markers of GI inflammation. While these tests are significant when elevated, a negative study may not necessarily exclude significant pathology, so several studies may be required to rule out inflammatory bowel issues. Calprotectin did not correlate well with ASD symptoms in one small study;[128] however, in the authors' ASD population, fecal calprotectin is frequently elevated above 50 mcg/g and is sometimes greater than 100 mcg/g.

◗ Intestinal permeability: Abnormal absorption of lactulose and mannitol, used to determine altered GI permeability, is a reported positive finding in over 40 percent of children with ASD.[129] However, this is not a consistent finding in the authors' practice population. Increased intestinal permeability could not be reproduced in another, albeit smaller, population studied.[130]

◗ Urinary organic acids: Thousands of these laboratory assessments have been performed on children with ASD at the authors' centers over the past 12 years and most demonstrate abnormalities in the citric acid cycle, which may be markers of mitochondrial dysfunction. Abnormal levels of citric acid and succinate are observed in most children when screened for urinary organic acids. Abnormally high levels of formiminoglutamic acid, which would be consistent with a functional folate deficiency (despite normal blood levels), and 3-methylhistidine (a metabolite of muscle catabolism in the presence of negative nitrogen balance) are commonly observed in patients prior to the onset of biomedical interventions such as nutritional supplementation, dietary changes, and medications. It is also common to find increased levels of methylmalonic acid[131] despite elevated serum B_{12}, which would seem to indicate impaired utilization of this vitamin.

Table 6. Biomarkers of Gastrointestinal Dysfunction

Biomarker	Clinical Significance
Calprotectin, eosinophil-X, S100A12	Markers of inflammatory bowel disease and food allergy related bowel inflammation, as well as infection or parasitosis
Intestinal permeability	Increased permeability consistent with bowel inflammation
Organic acid testing	Can indicate functional vitamin B12 and folate deficiency as well as intestinal dysbiosis
Gluten intolerance testing (Celiac panel)	Could indicate a need for further formal testing for celiac disease
Food allergy panel	Removal of allergic foods associated with improved autistic symptoms in some children
Stool culture and microscopic evaluation	Can identify intestinal dysbiosis, parasites, and pathogens

Several commercial laboratories offer organic acid testing with expanded evaluation beyond the typical testing. These expanded panels include investigation of bacterial and fungal metabolites that would reflect intestinal dysbiosis. In the authors' clinical experience, elevated yeast and anaerobic bacteria biomarkers in the urine appear to correlate with clinical responses to antifungal and/or antibacterial interventions. This is supported by the observation that oral vancomycin has been demonstrated to create short-term behavioral improvement in 80 percent of ASD children studied.[132] Currently, a study has been approved and funded to investigate the clinical response from fluconazole, a commonly prescribed antifungal observed to have clinical benefits in ASD. In addition, quantitative analysis of bacterial DNA in the gut has been used in children with ASD[22] and will hopefully be routine in the future. The levels of beneficial bacteria compared to pathogenic bacteria in the GI tract are a major determinant of intestinal immune function.[133]

▶ Gluten intolerance: Individuals with celiac disease are more likely to develop neurological disorders such as ADHD and developmental delay, hypotonia, and learning disorders;[134] thus, a celiac panel (available from major medical laboratories) can be evaluated. A 2008 retrospective study of 150 children with ASD found a three-fold higher prevalence of celiac disease than in the general pediatric population. The investigators suggested all children with ASD should be screened for celiac disease, regardless of the presence of GI problems.[135]

▶ Food allergy: In an eight-week study of 36 children with autism, the elimination of allergic foods (determined by a positive skin test) led to significant improvements in autistic behaviors (p<0.05), and worsening of these behaviors when the allergic foods were reintroduced.[136] Serum IgE and IgG testing to specific food antigens may be helpful in some cases. Several commercial laboratories now offer large panels of IgG testing of various food antigens. Jenkins and Vickers studied this with a laboratory in the United Kingdom but found inconsistent and unreliable results.[137] Oehling et al, however, found *in vitro* IgG4 and IgE food testing helpful in atopic children and called skin testing into question for its decreased specificity.[138] This is an area that remains complex and controversial, while requiring the combined use of clinical skills, elimination and reintroduction food challenges, and appropriate laboratory interpretation to provide useful insights and interventions.

◗ Stool culture and microscopic investigation: Screens for parasites, yeast, and abnormal bacteria are particularly important in cases associated with unexplained diarrhea, bloating, anal itching or redness, reflux, or apparent abdominal pain. Treatment of abdominal pain in children with ASD has been shown to improve certain core autistic behaviors.[139]

Heavy Metal Biomarkers

While lead surveillance is well accepted in cases of mental retardation and certain at-risk populations, the threat posed by mercury receives less attention from most practitioners. This occurs despite cord blood evidence that one of six children in the United States is born with exposure to levels of mercury high enough to cause impairments in IQ.[140] Several studies report that lead exposure is associated with hyperactivity and ADHD[21,34,141-143] as well as ASD.[21,144] In a study of 277 children, hair lead levels significantly correlated with ADHD.[34] In another study of 4,704 children, 4.2 percent of whom had ADHD, blood lead levels of 2-5 µg/dL increased the risk of ADHD four-fold (95% confidence interval (CI): 1.2-14.0).[35] In a study of 150 children with blood lead levels under 3.5 µg/dL, lead levels were significantly higher in children with ADHD compared to controls (p<0.05) and were significantly associated with hyperactivity and impulsivity.[143]

In a study of 52 children with ADHD and 59 typically developing children, mean blood mercury levels were associated with ADHD; a blood mercury level above 29 nmol/L was associated with a 9.7-fold (95% CI: 2.6-36.5) increased risk of ADHD.[145] With specimens collected in the late 1990s through 2001, Bradstreet et al demonstrated a six-fold increase in mercury after a three-day provocation with dimercaptosuccinic acid (DMSA, a chelator of lead and mercury) in 221 children with ASD compared to 18 age-matched typically developing children (p<0.005).[19] In a later but smaller study of a three-dose DMSA provocation followed by 24 hours of collection, Soden et al claimed to find no difference between children with autism and controls in urinary output of heavy metals.[146] However, given the small population in this study (15 children with ASD compared to 221 in the Bradstreet et al study) and concerns regarding the statistical methods, it is difficult to assess the significance of the Soden study. In a separate case report, exposure to mercury from a broken thermometer was associated with the development

of autistic features in one child.[147] Furthermore, several epidemiological studies correlate environmental mercury exposure with the prevalence of ASD.[148-151] More recently, Adams et al demonstrated a significant correlation between the relative level of urinary excretion of heavy metals, including mercury, after a DMSA provocation and the severity of autism.[17]

Table 7. Biomarkers of Heavy Metals

Biomarker	Clinical Significance
Blood lead	Increased levels associated with recent exposure
Packed RBC test	Can reflect recent exposure to toxic metals such as mercury, arsenic, lead, and others
Urinary fractionated porphyrins	Increased levels associated with increased body burden of toxic metals and other xenobiotics
Heavy metal challenge	Increased urinary output of metals after chelator administration associated with increased body burden of toxic metals

In situations where there is chronic, low level exposure to heavy metals, a history of exposure and clinical signs and symptoms are the key features of diagnosis. Past heavy metal intoxication is difficult to establish with present blood, hair, or urinary levels, since metals quickly move into preferred target organ sites like the brain, liver, heart, and kidneys, as is the case with organic mercury. Despite this diagnostic dilemma, the following biomarkers, summarized in Table 7, may be useful to assess heavy metal body burden.

◗ Lead: Blood lead levels should be tested in children with ASD who also have pica.[152] Furthermore, given the association between lead and an increased risk of ADHD, children with ADHD should also be tested. Intellectual impairment in children with blood lead concentrations below 10 mcg/dL has been documented.[153] This would seem to indicate any lead exposure is a potential threat to the IQ of developing children. Since it is well accepted that lead leaves the blood fairly rapidly to deposit into organs and the bone matrix, blood levels only indicate relatively recent

environmental exposure[154] and blood or urine porphyrin levels may be better indicators of past exposure.[155]

▸ Packed RBC levels of minerals and toxic metals (especially mercury, lead, and arsenic): RBC levels reflect ongoing exposure or rapid turnover from tissue reservoirs, as is the case when lead from prior exposure is liberated from bone during bone growth spurts. Rather than reflecting past exposures, these measurements tend to reflect the child's current environmental exposures and relative efficiency of naturally eliminating these heavy metals. A full mineral and metal panel test of PRBCs is helpful since it measures nutritional minerals as well as toxic metals.

▸ Urinary fractionated porphyrins: These molecular precursors of the heme structure have been found to be abnormally elevated in five studies of children with ASD[18,20,156-158] and are suitable to assess the current body burden of metals.[159] Most commercial laboratories are not set up to determine precoproporphyrin (pCP, also known as ketoisocoproporphyrin or KICP) levels.[20,160] Increased pCP is the more sensitive indicator for mercury burden.[159] If porphyrins are elevated compared to controls, then a post-chelation challenge with a six-hour urine toxic metal assay as described by Bradstreet et al[19] should be considered. In the authors' experience, oxidative stress is further capable of triggering an abnormal prophyrin response, apart from apparent heavy metal intoxication.

▸ Heavy metal challenge: This test is performed with a six-hour urine collection for the determination of heavy metals following a dose of an appropriate metal chelator.[19] Six hours is typically long enough since most studies

demonstrate that the majority of urinary metal excretion after chelation occurs during the first six hours. Given the difficulty of collecting urine specimens from many children, a first morning urine collection after a bedtime dose of a chelator may be an alternative way to test relative body metal burden.

Biomarker Directed Treatment
General Concepts of Biomedical Interventions

The fundamental goal of any integrative medical intervention is to create an ideal physiological state for optimal functioning, healing, growth, and development. Defining and treating the medical conditions or co-morbidities of ASD or ADHD would be expected to lead clinicians to specific interventions. Equally, using these biomarkers would be expected to gauge the efficacy of selected therapies. This is the same logic that medicine applies to measuring serial blood glucose levels during insulin therapy for diabetes. It follows then (by example) that the detection of oxidative stress would lead to antioxidant therapy, and a finding of inflammation would lead to some form of anti-inflammatory therapy. The expectation would then be to monitor the abnormal biomarker(s) while adjusting therapy to normalize the abnormal physiology.

Five general areas should be considered for biomedical interventions (Table 8), for which there is a great deal of overlap and interaction: (1) detoxification, (2) restoration of healthy gut flora, (3) reduction of oxidative stress, (4) normalization of immune function throughout the body, and (5) supplementation with adequate nutrients and micronutrients as well as enzymes (when necessary) to ensure proper digestion.

Detoxification requires the elimination of environmental toxicants (e.g., heavy metals, petrochemicals, and other xenobiotics) from both the external and internal environment. This is a complex process beyond the scope of this article, involving elimination of dietary sources of mercury, lead surveillance and removal (within the home, school, or other frequented sites), and heavy metal chelation using one or more of the available substances known to bind metals in children with objective evidence of metal toxicity. The use of organic foods and the elimination of indoor pesticides are encouraged wherever possible as additional measures to reduce toxic exposure. As noted previously, Adams et al demonstrated a correlation between toxic metal burden and the

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Table 8. General Areas of Biomedical Intervention

Biomedical Intervention
Detoxification Restoration of healthy gut flora Reduction of oxidative stress Normalization of immune function Nutritional supplementation

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severity of autism.[17] In the later phases of that study, they also demonstrated significant reductions in many of the core features of autism over a short course of DMSA treatment.[161] The exact mechanism whereby a short course of DMSA treatment might improve autism symptomatology is uncertain, but it may involve changes in thiol metabolism or a reduction in oxidative stress.[162]

Biomedical interventions also focus on the creation of a healthy intestinal ecosystem. While this has not received much attention from mainstream medicine until recently, it has been a cornerstone of integrative medicine and naturopathy for decades. The internal ecosystem requires healthy flora and the elimination of pathogenic microbes. It also requires the ability to digest complex food molecules into simple mono-amino acid and monosaccharide forms so proper absorption can take place. For example, some investigators have noted improvements in children with ASD using probiotics[163] and digestive enzymes.[164]

The process of supporting individual health also requires the elimination of excessive free radicals (which lead to oxidative stress) and the simultaneous reduction of excessive immune activation, which is often the driving force for free radical production. Multiple studies have shown antioxidants such as vitamin C,[165] carnosine,[166] carnitine,[167] and methylcobalamin injections along with folinic acid[96] improve certain behaviors in children with ASD. Likewise, some antioxidants, including pycnogenol,[168] carnitine,[169] and zinc[170] improve behaviors in children with ADHD.

Since a variety of physiological systems may be malfunctioning at the same time, the utilization of nutrients is often impaired at multiple levels. For this reason, the initial use of nutrient doses higher than the Recommended Daily Allowance (RDA) may be required. Once biochemical systems begin to function normally and inflammation and oxidation are normalized, supplementation can usually be reduced to more traditional levels. For example, studies in children with ASD[171] and ADHD[172] report behavioral improvements with the use of a multivitamin/mineral complex.

Given the overwhelming significance of potential CNS inflammation in many children with autism, developing an effective interventional strategy for this condition is a priority. Unfortunately, no therapeutic approach has been documented to reduce brain inflammation in ASD. However, Chez et al reported that children with

autism who received autoimmune treatments (such as steroids or IVIG) had much lower CSF TNF-α levels compared to children who did not receive such treatments.[114] Despite the lack of rigorous scientific investigation, the need to treat CNS inflammation justifies reasonable efforts to abate the disease process. Families should be given appropriate informed consent for any potential innovative approaches. In the authors' clinical experience and in the published literature, the use of anti-inflammatory medications[173,174] and other novel immune-modifying agents (e.g., IVIG) appear beneficial for use in many cases of ASD.[108,109,175,176] Furthermore, an integrative treatment plan may draw on numerous natural substances (e.g., curcumin – a well defined anti-inflammatory). The biomarkers discussed in this article can help establish and monitor the sufficiency of the treatment regimens selected by the practitioner.

An in-depth discussion of the biomedical treatments available for treating ASD is beyond the scope of this article, but a recent review outlines many of the potential biomedical treatment options in ASD including nutritional supplementation, diet, medication, and non-biological treatments.[175]

Conclusions

Both ASD and ADHD are currently diagnosed using only behavioral criteria. This article reviews evidence that ASD is a multifaceted biomedical disorder characterized by oxidative stress, decreased methylation capacity, limited transsulfuration production of cysteine and GSH, mitochondrial dysfunction, intestinal dysbiosis, increased toxic metal burden, cerebral hypoperfusion, and complex immune dysregulation characterized by both a unique inflammatory bowel disease and activation of neuroglial cells. Children with ADHD also share many of these same underlying features. It seems clear that successful treatment of ASD and ADHD requires clinicians to utilize a holistic approach that considers nutritional deficits, biochemical disruption, toxic exposures, and immunological abnormalities. The biomarkers discussed in this article are useful to guide the selection, efficacy, and sufficiency of biomedical interventions, which would likely include nutritional supplementation, dietary changes, and specific medications for treating GI pathogens and reducing inflammation.

Conflict of Interest Declaration

Acknowledgement

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