Clinically relevant biomarkers in 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 disorders. They are diagnosed based solely upon behavioral criteria with little to no consideration for potential biomedical underpinnings. Newer evidence, however, reveals that ASD is characterized by oxidative stress, decreased methylation capacity, limited production of glutathione, mitochondrial dysfunction, intestinal dysbiosis, increased toxic metal burden, immune dysregulation with a unique inflammatory bowel disease and immune activation of neuroglial cells, and ongoing brain hypoperfusion. Oxidative stress and immune dysregulation are also common features present in children with ADHD. These medical conditions have synergistically negative effects on development, cognition, focus, and attention. It is likely these biological abnormalities contribute significantly to the behavioral symptoms intrinsic in these disorders. Treatment for the underlying medical disorders is medically justified even if no clear immediate behavioral improvement is observed. In this article, we review the medical literature and discuss our clinical experience using various biomarkers for measuring oxidative stress, methylation capacity and transsulfuration, immune function, gastrointestinal problems, and toxic metal burden. These biomarkers are useful to guide the selection, efficacy, and sufficiency of biomedical interventions. The use of biomarkers is of greater importance in young children and individuals of any age with ASD, because they typically cannot adequately communicate regarding their symptoms.

Background

Autism (Autistic Disorder), Asperger Syndrome and Pervasive Developmental Disorder—

Not Otherwise Specified (PDD-NOS) comprise a heterogeneous spectrum of neurodevelopmental

disorders collectively termed autism spectrum disorders or ASD. All are behaviorally-defined and

characterized by restrictive and repetitive behaviors along with impairments in communication and

social interaction (1). The number of children diagnosed with ASD has substantially increased over

the last decade (2-4) and ASD currently affects as many as 1 out of 150 individuals in the United

States (U.S.) (5). However, since ASD occurs four times as frequently in males as females (6),

reporting the prevalence of ASD in all children will significantly underestimate the number of

affected males. A reasonable extraction of the overall data when applied to the male population

finds that 1 in 93 males (54 per 10,000) are likely affected with ASD (7) and the prevalence of

affected males approaches 2% of the general population in some studies (8, 9). ASD is traditionally

considered a "static" encephalopathic disorder (10) without any known cure and few proven

effective biomedical interventions. Furthermore, attention-deficit hyperactivity disorder (ADHD)

affects 4-12% of school age children (11) and is behaviorally characterized by features of

inattention, hyperactivity, and impulsivity (1). While both ADHD and ASD present complex

medical problems for physicians, a treatment approach can be streamlined for many children as a

result of advances in biomarker research. Given the large number of affected children with ADHD

and ASD and the continued increase in prevalence of both disorders, a simplified treatment

approach is needed for broad application to make it reasonable for pediatricians and family

practitioners to implement it in their practice.

Recent evidence has revealed that many children with ASD have multiple medical problems including: increased oxidative stress (12-14), decreased methylation capacity and limited transsulfuration (13, 15), mitochondrial dysfunction (7, 16), increased toxic metal burden (17-20), abnormal intestinal flora (dysbiosis) skewed toward an overgrowth of *Clostridia* species (21-23), immune dysregulation with a unique inflammatory bowel disease and immune activation of glial cells in the brain (24-27), and central nervous system (CNS) hypoperfusion or abnormal regulation of blood supply to the brain (28, 29). Furthermore, some of these medical problems have also been described in children with ADHD, especially oxidative stress (30-32). An informal review of nearly 4,000 records of children with predominately ASD diagnoses evaluated at our centers 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 for years to come. Given the broad array of pediatric specialties typically involved in these disorders, e.g. neurology, psychiatry, gastroenterology, immunology, and toxicology, it becomes a daunting task for any one medical provider to align the skills and expertise necessary to integrate appropriate care.

Complicating the necessary medical work-up and intervention is a hostile insurance environment wherein most insurers contractually exclude care for ASD. It is equally unclear how insurers might interpret their obligation to cover the expenses of medical evaluation for these many medical issues when they co-occur, precipitate, and/or exacerbate ASD. It seems to be a generally held misconception within the medical community that little is known about the underlying pathophysiology of these disorders or further, how to go about diagnosing these conditions or

treating them once uncovered. It should be emphasized that the diagnostic criteria for ASD and ADHD are based on behavioral symptoms, and apart from Fragile X and rubella as potential causes of ASD, very little is discussed in the DSM-IV-TR® regarding etiology or biology. Nor would it be reasonable to conclude that the mere presence of behavioral symptoms precludes the recovery from or diminishing of those symptoms through the treatment of underlying pathophysiologies. In fact, recovery from autism, while not widely published, is commonly observed. Consistent with our observations of recovery assisted by biomedical interventions, O'Hara and Szakacs recently published recovery from autism in one child (33).

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

Researchers have been examining the use of biomarkers in children with ASD for over 20 years (34). 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" (35). These biomarkers do not need to be exclusive to any disorder. As an example, oxidative stress has been reported as a common feature of vascular

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diseases (36) as well as many disorders of the CNS including ASD, schizophrenia, Alzheimer's disease, HIV-dementia, and Parkinsonism (37, 38). Oxidative stress therefore represents a common pathological pathway intrinsic in the creation of diverse clinical conditions, but which 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 at least some features of these disorders or at least prevent or slow their progression. The list of biomarkers described in this article is not meant to be exhaustive or all inclusive, but is intended to target the core biomedical issues frequently observed in children with ASD or ADHD. The use of biomarkers is of greater importance in young children and individuals of any age with ASD, because they typically cannot adequately communicate regarding their symptoms. As with any medical diagnostic evaluation, clinicians must rely on the history, physical examination, and all relevant biomarkers to properly diagnose and treat their patients.

Basic biomarkers

Several abnormalities have been described in children with ADHD and ASD which can be screened with simple laboratory tests:

1) CBC: A complete blood count (CBC) with differential can be performed. Abnormalities described in some children with ASD include a high blood monocyte count (39) and abnormal lymphocyte function (40-43).

2) CMP: A comprehensive metabolic panel (CMP) which includes liver and kidney testing is helpful. High albumin has been described in some children with ASD (44). 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 (45, 46).

3) Magnesium (Mg): Mg can be measured by any standard laboratory. In children with ADHD,

magnesium deficiency is common, occurring in up to 95% (47). In a six-month controlled study of

75 children with ADHD and magnesium deficiency (documented by low serum and red blood cell

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 of

the children compared to 25 children who did not receive magnesium ($p < 0.05$) (48). In a 6-month

controlled study of 33 children with ASD, the use of vitamin B6 (0.6 mg/kg/day) and magnesium (6

mg/kg/day) led to significant reduction of autistic symptoms ($p < 0.0001$) in 70% of the children,

including social interaction, communication, and stereotypies. No adverse effects were observed.

However, when the B6/Mg treatment was stopped, the undesired behavior returned within several

weeks (49).

4) Zinc (Zn): Zn 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 ($p < 0.001$) (50). Other investigators studied a different 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) (51). In a controlled study of 45 children with autism compared to

41 typically-developing children, the plasma and erythrocyte zinc levels were significantly lower in

the autism group ($p < 0.05$) (52).

5) Other minerals: One study reported that children with ASD and pica had lower chromium hair levels (53). Low hair iodine and lithium levels have also been described in some children with ASD (53). One study of 20 children with autism and 15 typically-developing children reported significantly lower red blood cell selenium ($p < 0.0006$) in the autism group (54). A reasonable method of determining mineral content is to assess packed red blood cell (PRBC) element concentrations, and this technique has been evaluated in the pediatric population (55).

6) Iron: Iron deficiency appears to be relatively common in ADHD (56) and serum ferritin is low in many children with ADHD compared to typically-developing children (57, 58). In addition, iron deficiency is common in ASD and serum ferritin is low in many children with ASD (59, 60). In a randomized, double-blind, placebo-controlled study of 23 children with ADHD who had serum ferritin levels less than 30 ηg/ml, supplementation with ferrous sulfate (80 mg/day) over a 12-week period was well-tolerated and significantly improved ADHD symptoms ($p < 0.008$). No improvements were observed with a placebo (61). In an 8-week open-label study of 33 children with ASD, supplementation with iron (6 mg/kg/day) significantly improved sleep and increased mean ferritin levels. The investigators suggested that children with ASD should be routinely screened for iron deficiency (62). We recommend obtaining a ferritin and serum iron level.

7) Hypothyroidism: Hypothyroidism has been described in some children with ASD (63) and ADHD (64) and therefore screening for hypothyroidism with a blood test for TSH is recommended.

8) Cholesterol: A subset of children with ASD have abnormally low cholesterol levels with one study demonstrating that 19% of children with ASD had a cholesterol level below 100 mg/dL (65).

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 (66).

9) Testosterone: A small percentage of children with ASD may have elevated testosterone levels (67) and elevated fetal testosterone levels also appear to be associated with a higher likelihood of developing ASD (68). Thus measuring levels of serum testosterone and related androgens may be indicated. In our 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 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 children with ASD (13, 14, 37) and ADHD (30-32). Reduced glutathione is the primary intracellular antioxidant and has been shown to limit mercury-induced neurotoxicity (69). Impaired glutathione (GSH) production contributes to oxidative stress which may delay the clearance of heavy metals and certain xenobiotics (70). In two prospective studies, over 50% of children with ASD had significantly lower plasma levels of GSH and cysteine ($p < 0.001$ for both) when compared to typically-developing children (13, 15). 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" (13). The following biomarkers can be measured to assess the level of oxidative stress:

1) Reduced and oxidized GSH (13): An internet search of laboratory providers for this special

testing found several commercially available companies capable of measuring these valuable

markers. Measuring total GSH with oxidized (GSSG) and/or reduced GSH will help determine the

patient's oxidation status.

2) 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 and both are

significantly decreased in children with ASD compared to typically-developing children (14, 71)

and in one study lower levels of both of these proteins was associated with regression and loss of

previously-acquired language skills (71). We would caution, however, that a variety of conditions

influence the levels of either protein, thus making interpretation challenging.

3) Blood ammonia and lactate: Ammonia is derived from the deamination of the amine group of

amino acids either by gut bacteria or by the liver. The process of detoxifying ammonia via the urea

cycle is metabolically expensive and costs the body three valuable, high-energy ATP molecules for

every ammonia molecule processed. Hyperammonemia is more toxic for children than adults and

can lead to permanent damage to the CNS (72). Lactic acid is a by-product of the anaerobic

metabolism of glucose. Typically, clinicians look for serum lactate levels greater than 3 mm/L for

support of mitochondrial disease (73), although lactate levels can be normal in some mitochondrial

diseases (74). Lactate is elevated in a variety of disorders other than ASD, but levels above 2 mm/L

support mitochondrial dysfunction if proper sampling techniques are followed. If at all possible,

lactate and ammonia levels should be drawn without a tourniquet after the venipuncture or IV is

started. Ideally, the child should not be fighting during the process and may require sedation to get

accurate results. Increased lactate levels may need confirmation with a separate blood draw.

Elevations in either ammonia or lactate likely reflect a state of mitochondrial hypofunctioning in ASD (16, 75, 76) and are standard tests done at all hospitals. The blood used for ammonia and lactate testing requires immediate icing once placed in the specimen tubes.

4) Serum carnitine profile: Carnitine levels are often reduced in children with ASD (16) and may reflect mitochondrial dysfunction. We consider this test routine for any child with hypotonia or other signs and symptoms of mitochondrial dysfunction.

5) Urinary 8-hydroxyguanine (8-OHG): This is a marker of RNA oxidation in the mitochondria and cytoplasm of cells and is an easily obtained urinary marker useful for evaluating intracellular oxidative stress (77). The DNA marker of oxidative stress (8-dOHG) is not elevated in most cases of ASD (78) but is in some children with ADHD (32). These tests are available from Laboratoire Philippe Auguste (Paris, France).

6) Urinary isoprostane is a marker of fatty acid oxidation and reflects cell membrane (extracellular) oxidative stress, and is elevated in many children with ASD compared to controls (78). This test is available from Laboratoire Philippe Auguste (Paris, France).

7) Vitamin D has emerged as a newer concern among some practitioners. It is interesting to note that vitamin D deficiency and autism share the common qualities of enlarged brain size and enlarged ventricles (79, 80). We have also seen increased rates of autism in some darker-skinned populations and insufficient vitamin D may be partly responsible (81). Vitamin D status is important to consider because of its role in reducing oxidative stress through both GSH production and serving as an antioxidant itself (82-84). We have been using a vitamin D panel which is

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available from many commercial laboratories. When evaluating a potential deficiency state, a decrease of the 25-hydroxycalciferol form is diagnostic of inadequate dietary intake.

Methylation capacity and transsulfuration biomarkers

Methylation and transsulfuration pathways represent core areas of metabolic activity vital to all life. These connected and interdependent pathways generate required methyl-donors via the conversion of methionine to S-adenosylmethionine (SAM or SAMe) which in turn donates its methyl group to catecholamine neurotransmitters, cell membranes, DNA, and other body chemistry. The end product is homocysteine which is merely demethylated methionine. Excess homocysteine is required to generate cysteine which is the rate-limiting step for the production of the vital and dominate intracellular anti-oxidant, reduced GSH. An oxidized intracellular condition would inhibit the methionine cycle, making the reducing capacity of GSH critical to its own production. It is very well-accepted that neurons are extremely sensitive to oxidation and this makes reduced GSH essential for neuronal survival (85). James et al. have documented methylation and transsulfuration disruption in the majority of children with ASD (13, 15, 86). Deficiency, as measured by decreased levels of fasting plasma cysteine or its dimer (cystine), may predict improvement with methylcobalamin injections and/or folinic acid (15). 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 can be checked to assess methylation and transsulfuration pathways:

1) 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.

2) Fasting plasma methionine: Methionine is an essential amino acid and is the main methyl donor via the intermediary S-adenosylmethionine.

3) Plasma sulfate: Sulfate is often low in children with ASD (87). Several studies have reported that the conjugation of sulfate to acetaminophen (an indicator of deficient phase II hepatic sulfation-detoxification) is impaired in children with ASD (88-90) which in turn may impair the detoxification of many chemicals and metabolites, such as phenolic xenobiotics, which would adversely impact brain function (91).

Immune biomarkers

It is very difficult to get a direct measurement of brain inflammation, and even cerebral spinal fluid (CSF) studies have offered inconsistent findings. Given the literature demonstrating the presence of both cerebral oxidation and inflammation in ASD, we look for a pattern of up-regulation of cellular immunity combined with other features of immune dysregulation to form a clinical picture.

1) Serum autoantibodies to brain endovasculature (92, 93): This test is performed exclusively at the Neuromuscular Laboratory at Washington University in St. Louis. Details related to specimen handling and requirements are available from their website. In our practice, the presence of either IgG or IgM antibodies to brain endovasculature is common (approaching 2/3 of cases of ASD) and

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predicts speech delay. 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. (27).

2) Neopterin and biopterin: These biomarkers are often elevated in both the urine and in monocytes of children with ASD compared to typically developing children (39, 94, 95). Neopterin predicts the degree of cell-mediated immune activation and biopterin is a measure of the immune system's attempt to compensate for the oxidative stress which is induced by immune activation. Laboratoire Philippe Auguste (Paris, France) is the only commercial laboratory we are aware of with pediatric controls for urinary studies. We observe a significant correlation between elevated urinary neopterin and favorable clinical responses to immune interventions.

3) Immunoglobulin subsets: IgG (subclasses 1-4), IgM, IgA, and IgE. High IgG, IgG2, and IgG4 levels have been described in a subset of children with ASD (44). While this does occur, our experience parallels that of Gupta et al. with deficiencies of IgG subclasses, IgA, and IgM along with specific cellular immune deficiencies all being more common than increased levels of globulins (96). Oleske observed that in a subset of children with ASD, immune deficiency predicted a favorable response to intravenous immunoglobulin (IVIG) therapy (97). IgE is elevated in some children with ASD (98, 99) and IgA is low in a subset of children (98, 100). Extreme IgA deficiency is rare, but is important to exclude prior to starting IVIG as the treatment of a child who is extremely IgA-deficient requires special product selection to prevent anaphylaxis (101).

4) Vaccine titers: These become useful in defining specific antibody deficiency 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.

5) Antinuclear Antibodies (ANA): These are known to reflect autoimmunity and are elevated in a subset of children with ASD (93).

6) Urinary N-Methylhistamine: This may be useful in some cases of ASD and is a biomarker of significant inflammatory bowel disease (102). This test is also elevated in asthma. Mayo Medical Laboratories (Rochester, MN) and Specialty Laboratories (Santa Monica, CA) are capable of performing this test.

7) Tumor Necrosis Factor (TNF)-alpha: Chez et al. observed a markedly increased ratio of CSF/ Serum TNF-alpha compared to controls (103). This is an intriguing observation which could represent an ideal way to assess the inflammatory state of the CNS. While invasive, it is probably a test which deserves more attention and greater efforts despite the expected poor parental acceptance of CSF testing.

8) Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infection (PANDAS): Giedd et al. first reported PANDAS in 1996 (104). 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 NIMH group studying this found streptococci (strep) induced autoantibodies to basal ganglion. This is much the same as the way the immune system forms cross-reactive antibodies between GABHS and heart valves in rheumatic heart disease. We do frequently observe both OCD and tic exacerbations in the ASD population, but cultures are often negative or there is no clear antecedent strep infection. This makes the diagnosis of PANDAS complicated since, apart from the clinical picture of a positive throat culture preceding the onset of new OCD and tic behaviors, there is no clinically useful diagnostic testing at this time.

The basal ganglion autoantibody tests are not commercially available and traditional antibody testing for strep (antistreptolysin-O and anti-DNase B) can be deceptive since many healthy children will carry high titers for a long time after infection or during strep outbreaks at school (personal communication from Sue Swedo, MD, NIMH). Treatment for this is controversial, but prolonged courses of antibiotics have been proposed (105) and in our experience may be helpful in select ASD-PANDAS cases. The group at NIMH has also investigated the use of novel treatments including immunomodulatory therapies such as therapeutic plasma exchange (TPE) and intravenous immunoglobulin (IVIG) (106). Our group has no experience with TPE, however we find monthly infusions of IVIG are needed for extended periods of time in order to see improvements when ASD and PANDAS co-occur.

Gastrointestinal biomarkers

Gastrointestinal inflammation has been described in many children with ASD (26, 107, 108). Other GI problems (reflux, constipation, food sensitivities, and abnormal flora) are also common. For example, Horvath et al. reported in a study of 36 children with autism that reflux esophagitis was present in 69%, chronic gastritis in 42%, chronic duodenitis in 67%, and low intestinal carbohydrate digestive enzyme activity in 58% (109). Another study of 50 children with ASD and 50 typically-developing children reported that 70% of the ASD group had a history of GI problems compared to 28% of the control group (p < 0.001) (110). Common GI problems found in one study of 112 children with autism included diarrhea (28%), gaseousness (60%), bloating (38%), abdominal pain (38%), and fecal impaction (19%). Importantly, 80% 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 (111). Several studies have also reported dysbiosis in children

with ASD, including significant overgrowth of *Clostridia* species (21-23) and yeast (*Candida albicans*) (112) in the GI tract compared to typically-developing children. The following biomarkers can be obtained to assess these problems:

1) Fecal testing: Calprotectin (113, 114), eosinophil X (115), and S100A12 (116) are all markers of inflammation in the GI tract. These tests are significant when elevated, but 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 (117). In our population, however, fecal calprotectin is frequently elevated above 50 mcg/gm and commonly greater than 100 mcg/gm.

2) Intestinal Permeability: Abnormal absorption of lactulose and mannitol can be used to determine altered permeability in the gastrointestinal tract and this test is abnormal in over 40% of children with ASD (118). However, this is not a consistent finding in our practice population. Increased intestinal permeability could not be reproduced in another, albeit smaller, population studied (119).

3) Urinary organic acids: Literally thousands of these studies have been performed on children with ASD at our centers over the past 12 years and most demonstrate abnormalities in the citric acid cycle. We observe abnormal levels of citric acid and succinate in most children when we screen for urinary organic acids. Abnormally high levels of formiminoglutamic acid (FIGLU), which would seem to indicate 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 our 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 (120)

despite elevated serum B12, which would seem to indicate impaired utilization of this vitamin.

Several commercial laboratories offer organic acid testing (OAT) with expanded evaluation beyond

the typical organic acid testing. These expanded panels, include investigation of bacterial and

fungal metabolites which would reflect abnormal intestinal flora (dysbiosis). In our clinical

experience, elevated yeast and anaerobic bacteria biomarkers in the urine do seem 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% of ASD children studied (121). Currently, a study has been approved and

funded to investigate the clinical response from fluconazole, which is a commonly prescribed

antifungal observed to have clinical benefits in ASD. Additionally, quantitative analysis of bacterial

DNA in the gut has been used in children with ASD (21) and will hopefully be routine in the future.

The levels of beneficial compared to pathogenic bacteria in the GI tract are considered a major

determinant of intestinal immune function (122).

4) Gluten intolerance: Individuals with celiac disease are more likely to develop neurological

disorders such as ADHD and developmental delay, hypotonia, and learning disorders (123), and

thus a celiac panel (available from all major medical laboratories) can be evaluated. Recently, a

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 that all children with ASD

should be screened for celiac disease, regardless of whether or not GI problems are present (124).

5) Food allergy: In an 8-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 (125). Serum IgE and

IgG testing to specific food antigens may be helpful in selected cases. Several commercial laboratories now offer large panels of IgG testing to various food antigens. Jenkins and Vickers have studied this with a laboratory in the UK and found inconsistent and unreliable results (126). Oehling et al. however, found *in vitro* IgG4 and IgE testing helpful in atopic children and called skin testing into question for its decreased specificity (127). This is an area that remains complex and controversial while requiring the combined use of clinical skills, elimination/reintroduction food challenges, and appropriate laboratory interpretation to provide useful insights and interventions.

6) Stool culture: This test screens for parasites, yeast, and abnormal bacteria. This is 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 (128).

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 much less attention from most practitioners. This occurs despite cord blood evidence that 1 out of 6 children are born exposed to levels of mercury high enough to cause impairments in IQ (129). Several studies have reported that lead exposure is associated with hyperactivity and ADHD (20, 130-133) as well as ASD (20, 134). In a study of 277 children, hair lead levels were significantly correlated with ADHD (132). In another study of 4,704 children, 4.2% had ADHD, and blood lead levels between 2-5 µg/dL increased the risk of ADHD by 4-fold (95% confidence interval (CI) of 1.2 – 14.0) (135). 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 (133). In a study of 52 children with ADHD and 59 typically-developing children, mean blood mercury levels were also associated with ADHD; a blood mercury level above 29 ηmol/L was associated with a 9.7-fold (95% CI of 2.6 – 36.5) increased risk of ADHD (136). Bradstreet et al. demonstrated nearly a six-fold increase in mercury after a three-day provocation with succimer (a chelator of lead and mercury) in 221 children with ASD compared to 18 age-matched typically-developing children (p < 0.005) with specimens collected in the late 1990's through 2001 (17). In a later but much smaller study of a three-dose DMSA provocation followed by 24 hours of collection, Soden and colleagues showed no difference between children with autism and controls in urinary output of heavy metals (137). Given the very small population in this study (15 children with ASD compared to over 200 in the Bradstreet et al. study), it difficult to assess the significance of the Soden et al. study. In a separate case report, exposure to mercury from a broken thermometer was associated with the development of autistic features in one child (138). Furthermore, several epidemiological studies have also correlated environmental mercury exposure with the prevalence of ASD (139-142).

In situations where there is low-level, but chronic 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, we find the following biomarkers may be useful to assess heavy metal burden:

1) Lead: Children with ASD who also have pica should be screened for lead exposure with a blood lead level (143). Furthermore, given the association between very low lead levels and increased risk of ADHD, children with ADHD should a blood lead level drawn. Intellectual impairment in children with blood lead concentrations below 10 mcg per deciliter has been documented (144). 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 in organs and bone matrix, blood levels only indicate relatively recent environmental effects (145). As such, blood or urine porphyrin levels may be better indicators of exposure (146).

2) Packed erythrocyte levels of minerals and toxic metals (mercury, lead, and arsenic in particular): Erythrocyte 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. These measurements tend to reflect the child's current environmental exposures and perhaps their relative efficiency of naturally eliminating heavy metals much more than they support past exposures. A full mineral and metal panel test of packed erythrocytes (PRBC) is helpful since it measures nutritional minerals as well as toxic metals.

3) Urinary fractionated porphyrins: These molecular precursors of the heme structure have been described as being abnormally elevated in five separate studies of children with ASD (18, 19, 147-149) and are suitable to assess the current body burden of metals (150). Most commercial laboratories are not set-up to determine precoproporphyrin (pCP, also known as ketoisocoproporphyrin or KICP) levels (18, 151). Increased pCP is the more sensitive indicator for mercury burden (152). 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. (17) may be helpful and should be considered.

4) Heavy metal challenge: This is performed with a six-hour urine collection for the determination of heavy metals following a dose of an appropriate metal chelator [17]. We typically choose six hours as most studies demonstrate that the majority of urinary output of metals after chelation occurs during the first six hours. A first morning urine collection after a bedtime dose of a chelator may be an alternative way to test relative body metal burden in some children.

Biomarker directed treatment

General Concepts of Biomedical Interventions. The simple goal of any integrative medical intervention is to create an ideal physiological state for optimal functioning, healing, growth, and development. Defining the medical conditions or comorbidities would be expected to lead to numerous reasonable interventions. Equally, using these biomarkers would be expected to gauge the efficacy and sufficiency of those interventions. This is the same logic that medicine applies to measuring serial blood glucose levels during insulin therapy for diabetes. It follows (by example) that the detection of oxidative stress would lead to antioxidant therapy, and finding inflammation would lead to some form of anti-inflammatory therapy. The expectation would then be to follow the abnormal biomarker(s) while adjusting therapy to normalize the abnormal physiology.

There are five general areas to be considered for biomedical interventions, for which there is a great deal of overlap and interactions. These are: 1) detoxification, 2) restoring healthy flora to the gut, 3) reduction of oxidative stress, 4) normalization of immune function throughout the body, and

5) supplementation with adequate nutrients and micronutrients as well as the enzymes necessary to ensure digestion.

Detoxification requires the elimination of environmental toxicants (e.g. heavy metals, petrochemicals, and xenobiotics) from both the external and internal environment. This is a complex process beyond the scope of this paper which involves 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 medications known to bind metals in children with objective evidence of metal toxicity.

Biomedical interventions also focus on the creation of a healthy intestinal ecosystem. While this has not received much attention from mainstream medicine until just 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 the complex molecules of food into their simple mono-amino acid and monosaccharide forms so proper absorption can take place. Some investigators have noted improvements in children with ASD using probiotics (153) and digestive enzymes (154).

The process of supporting the individual's health also requires the elimination of excessive free radicals (oxidative stress) and the simultaneous reduction of excessive immune activation which is often the driving force for free radical production. Multiple studies have shown that the use of antioxidants such as: vitamin C (155), carnosine (156), carnitine (157), and methylcobalamin injections (86) improve certain behaviors in children with ASD. Likewise, some antioxidants,

including pycnogenol (158), carnitine (159), and zinc (160) improve behaviors in children with ADHD.

Since a variety of physiological systems are malfunctioning at the same time, the utilization of nutrients is impaired at multiple levels. For this reason, the initial use of higher than RDA recommended levels of nutrients may be required. Once biochemical systems start to function normally and both inflammation and oxidation are normalized, supplementation can usually be reduced to more traditional levels. For example, studies in children with ASD (161) and ADHD (162) have reported 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 must be a priority. Unfortunately, no therapeutic approach has been documented to reduce brain inflammation in ASD. Despite this lack of rigorous scientific investigation, the need to treat CNS inflammation justifies reasonable efforts at abating the disease process. Families should be given appropriate informed consent for any potential innovative approaches. In our clinical experience and in the published literature, the use of anti-inflammatory medications (163, 164) and other novel immune modifying agents (e.g., IVIG) appear beneficial for use in many cases of ASD (97, 98, 165).

Conclusions

Both ASD and ADHD are currently diagnosed using only behavioral criteria. This paper reviewed 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 immune dysregulation characterized by both a unique inflammatory bowel disease and activation of neuroglial cells. It is clear that in order to successfully treat ASD clinicians need to utilize a holistic approach which considers as significant contributing factors: nutritional deficits, biochemical disruption, toxic exposures, and immunological abnormalities. In children with ADHD, nutritional deficiencies, oxidative stress, and immune dysregulation are also common features. The biomarkers discussed in this paper 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.

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