

Metabolic pathology of autism in relation to redox metabolism

An imbalance in glutathione-dependent redox metabolism has been shown to be associated with autism spectrum disorder (ASD). Glutathione synthesis and intracellular redox balance are linked to folate and methylation metabolism, metabolic pathways that have also been shown to be abnormal in ASD. Together, these metabolic abnormalities define a distinct ASD endophenotype that is closely associated with genetic, epigenetic and mitochondrial abnormalities, as well as environmental factors related to ASD. Biomarkers that reflect these metabolic abnormalities will be discussed in the context of an ASD metabolic endophenotype that may lead to a better understanding of the pathophysiological mechanisms underlying core and associated ASD symptoms. Last, we discuss how these biomarkers have been used to guide the development of novel ASD treatments.

Keywords: autism • folate • glutathione • methylation • mitochondria • oxidative stress • redox

Despite decades of research, we have limited knowledge of the causes of and treatments for autism spectrum disorder (ASD). ASD research effort has primarily concentrated on the genetic causes of ASD [1], despite the fact that inherited single-gene and chromosomal defects account for the etiology of ASD in only a minority of cases [2]. It is becoming clear that the etiology of ASD involves complicated interactions between genetic predisposition and environmental exposures or triggers. Indeed, a recent study of dizygotic twins estimated that the environment contributes a greater percentage of the risk of developing autistic disorder compared with genetic factors [3].

Recent studies have recognized that a broad range of children with ASD have impairments in several basic physiological processes such as energy generation systems [1], as well as folate [4,5], tetrahydrobiopterin [6,7] and glutathione redox [8–10] metabolism. The importance of identifying and understanding these physiological abnormalities cannot be understated. While most genetic mutations

are not treatable, treatments are available for metabolic disorders. Given the broad range of children with ASD that may be affected by such metabolic disturbances, targeted treatments have the potential to improve physiological function and health in a large number of children with ASD. Careful consideration of the physiological systems found to be disrupted in ASD demonstrates that they are tightly interconnected such that primary disturbance in one metabolic pathway can lead to dysfunction in the other interconnected metabolic pathways. Research from our laboratory and others suggests that a subset of children with ASD may have a metabolic phenotype in which the integrated pathways involving regulation of redox homeostasis is primarily impaired, with resulting secondary disruption of other important metabolic systems.

In this review, we outline the importance of redox regulation pathways and demonstrate how redox regulation is linked with other key metabolic systems. We review the evidence for a disruption in redox regula-

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tion as well as associated pathways in children with ASD, and identify the biomarkers that can be used to measure the function of these pathways as well as the consequences of dysfunction. Last, we demonstrate how these biomarkers can inform novel treatment strategies to correct these metabolic disturbances in order to improve core and associated symptoms of ASD.

Redox metabolism

Reactive species

Reactive species are chemically reactive molecules that possess an unpaired electron. Since molecules are most stable when electrons are paired, such reactive species will readily attract an electron from another molecule, causing it to become unstable in turn. The most common reactive species are molecules with oxygen and nitrogen atoms, called reactive oxygen species (ROS) and reactive nitrogen species (RNS). The presence of cellular reactive species can be either an advantage or disadvantage with respect to cellular health depending on the exposure level. A low level of reactive species is necessary to stimulate normal cellular metabolism and function. However, high levels of ROS/RNS can damage cellular membranes, lipids, proteins and DNA, leading to functional deficits. Importantly, the cell has a redundant set of protective enzyme systems designed to control the amount of ROS/RNS. When high levels of reactive species overwhelm these systems, a state of oxidative stress is induced.

Physiological importance of low-level reactive species

Subtle changes in redox status are essential for cell signaling, including cell cycle regulation, cell differentiation, enzyme activity regulation, transcription factor binding and gene expression, immune system activation, and regulation of mitochondrial function [11,12]. Reactive species are also important for neurodevelopment. For example, ROS are important for long-term potentiation, a process that is essential for learning and memory, and can stimulate pathways responsible for increasing synaptic strength as well as synaptic vesicle recycling [13]. In this way, subtle reversible shifts in redox equilibrium can act as a rheostat to maintain normal neuronal communication and connectivity, and also comprise an integrated cell response to environmental stressors.

Reactive species & disease

Oxidative stress occurs when cellular antioxidant defense mechanisms are unable to counterbalance the ROS/RNS generated from oxidative metabolism or pro-oxidant environmental exposures. The potential

role of oxidative stress in ASD pathogenesis has not been extensively investigated, despite knowledge of the developing brain's high vulnerability to oxidative damage [14]. Despite comprising only 2% of body weight, the brain utilizes more than 20% of oxygen consumed by the body. High energy demands from oxidative metabolism in addition a high concentration of polyunsaturated fatty acids and relatively low antioxidant enzyme activity are thought to render the brain more vulnerable to oxidative insult than most organs. Once the cell's capacity to buffer excessive ROS/RNS is exceeded, damage occurs to proteins, and lipid membranes and nuclear and mitochondrial DNA disrupt, leading to cellular dysfunction or apoptotic cell death.

Regulating reactive species & redox homeostasis through glutathione

Glutathione (GSH) is a tripeptide composed of cysteine, glycine and glutamate that is synthesized *de novo* in all cells of the body and serves as the major intracellular redox buffer. The sulfhydryl (SH) group of the cysteine moiety provides the reducing equivalents of the GSH molecule. This reducing environment is maintained inside the cell by the high ratio of reduced GSH to the oxidized disulfide GSSG [15]. The GSH/GSSG redox equilibrium regulates a pleiotropic range of functions, including free radical scavenging and redox homeostasis, maintenance of protein redox conformation and regulation of redox sensitive enzyme activity, cell membrane integrity and signal transduction, phase II detoxification, and regulation of proliferation, differentiation and apoptosis.

GSH can become depleted with conditions of chronic ROS, resulting in a disruption in redox homeostasis. Under normal physiologic conditions, the optimal GSH/GSSG redox ratio is maintained by the NADPH-dependent enzyme glutathione reductase (GR) which converts GSSG to GSH. However, when excessive oxidative stress exists, the cell exports GSSG in an attempt to regain intracellular redox homeostasis, thereby decreasing the total amount of GSSG available to recycle to GSH. In addition, GSH is a scavenger for cellular toxins. GSH is conjugated with toxins by the enzyme glutathione-S-transferase (GST), exported from the cell to the plasma and excreted in the urine in an attempt to protect cellular health. Through either of these cellular protective processes, the total intracellular GSH will be decreased in the cell, resulting in an increased requirement for *de novo* GSH synthesis [15]. The process of *de novo* GSH synthesis requires cysteine, the rate-limiting amino acid for GSH synthesis, and ATP, the product of mitochondrial metabolism.

Metabolic pathways linked to glutathione production

A diagram of the three interconnected pathways of folate, methionine and GSH metabolism found to be abnormal in many autistic children is presented in Figure 1 with a detailed biochemical description. Due to the mutually interdependent nature of these three pathways, genetic or environmental perturbation of folate or methylation metabolism will indirectly impact GSH synthesis and, conversely, alterations in GSH synthesis can alter flux through pathways of folate and methionine metabolism [16].

Methylation metabolism

Methylation metabolism is directly connected to GSH synthesis through homocysteine and cysteine. Methionine is necessary for the synthesis of *S*-adenosylmethionine (SAM), the major methyl donor for all cellular methylation reactions. SAM is essential in the regulation of gene expression and stability through its role in

DNA and histone methylation, as well as enzyme regulation as an essential cofactor for many SAM-dependent enzymes. The methyltransferase reaction results in the production of *S*-adenosyl-homocysteine (SAH), which is further metabolized to homocysteine and adenosine. Approximately 50% of homocysteine leaves the folate-dependent methylation cycle and is metabolized to cystathionine and cysteine. Importantly, cysteine is the rate-limiting amino acid for GSH synthesis. In this way, GSH synthesis is linked to adequate folate, methionine and SAM availability. Folate and methionine levels can be negatively affected by genetic and environmental factors that reduce folate availability and/or increase oxidative stress as methionine synthase is inhibited by excessive ROS/RNS [17].

Folate metabolism

Folic acid, the synthetic form of folate used to fortify food and in vitamin preparations, enters the folate cycle through a reduction by dihydrofolate reductase, yield-

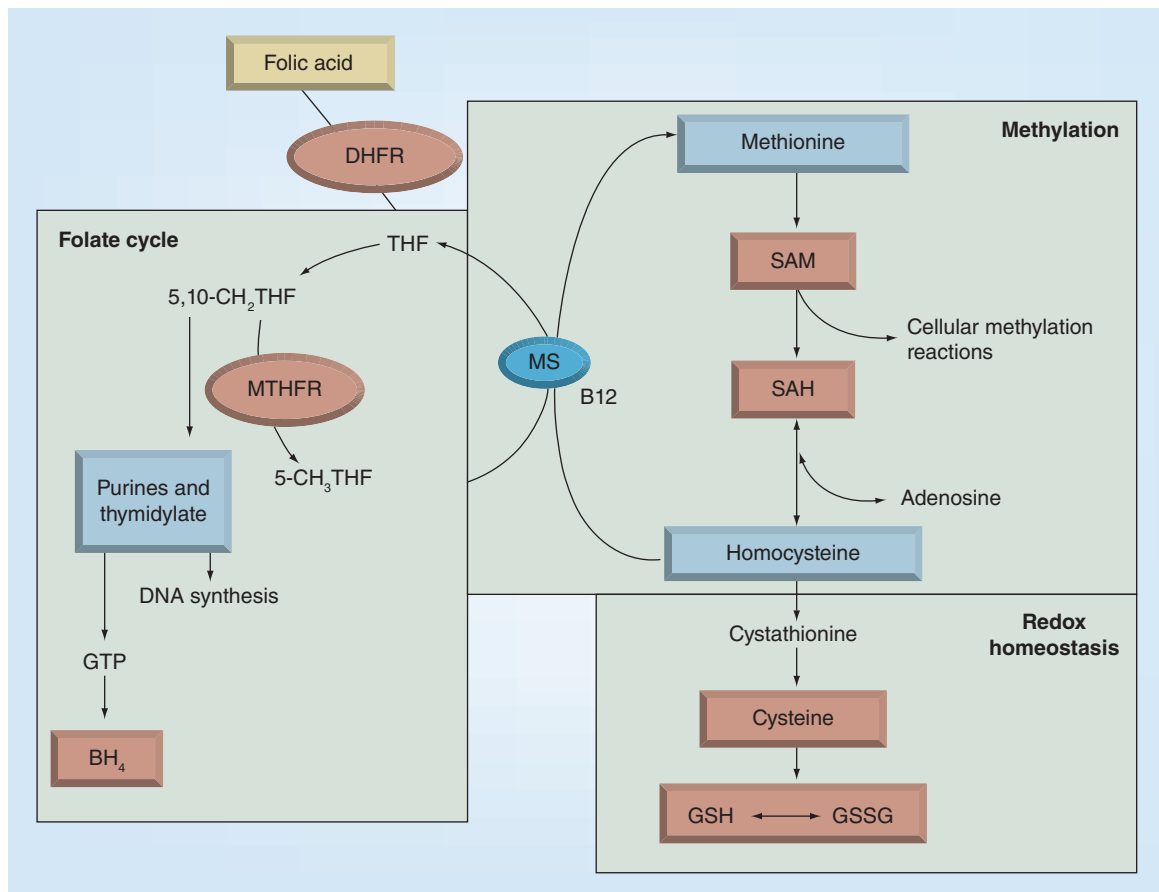


Figure 1. Three interconnected critical metabolic pathways that manifest abnormalities in individuals with autism spectrum disorder: folate, methionine and glutathione redox metabolism. Metabolites and enzymes highlighted in red are those found to be consistently abnormal in individuals with autism spectrum disorder.

BH₄: Tetrahydrobiopterin; DHFR: Dihydrofolate reductase; GSH: Reduced glutathione; GSSG: Oxidized glutathione; GTP: Guanosine-5'-triphosphate; MS: Methionine synthase; MTHFR: Methylene tetrahydrofolate reductase; SAM: *S*-adenosyl methionine; SAH: *S*-adenosylhomocysteine; THF: Tetrahydrofolate.

ing biologically active tetrahydrofolate (THF). THF serves as single carbon donors in any one of three oxidation states: 5-methyl-THF, 5,10-methylene-THF and 10-formyl-THF. The single-carbon donor 5-methyl-THF is used along with vitamin B12 (cobalamin) to support methylation capacity by converting homocysteine into methionine and methyl donor SAM. 5,10-methylene-THF is used for pyrimidine synthesis by participating in the conversion of deoxyuridine 5'-phosphate to deoxythymidine 5'-phosphate, as well as providing one-carbon groups for *de novo* synthesis of purines. Purines and thymidine are essential nucleic acids for DNA synthesis, repair and replication in all cells as well as precursors of other important cellular reactions.

Abnormalities in glutathione metabolism associated with autism spectrum disorder

Decreased GSH concentration and GSH/GSSG ratio, and increased GSSG concentration have been reported in plasma, peripheral blood mononuclear cells, lymphoblastoid cells lines, brain tissue and mitochondria of individuals with ASD [10,18–23]. Some studies have demonstrated a reduction in total GSH in plasma and whole blood, but this biomarker appears to be more variable than the other measures of GSH [18]. Although low plasma concentrations of GSH precursor metabolites, including cystathionine, cysteine and cysteinylglycine, have been reported, a decrease in plasma and intracellular concentration of cysteine is the only consistent finding across studies [18].

Autism-associated abnormalities that could disrupt glutathione metabolism

Abnormalities in glutathione pathways

GSH functions as an antioxidant primarily by converting hydrogen peroxide into water. This reaction is catalyzed by the enzymes glutathione peroxidase (GPx), a selenium-dependent enzyme. GPx activity has been examined in the plasma, whole blood, erythrocytes and platelets of individuals with ASD. Although its activity has been reported to be increased in some studies and decreased in others, a meta-analysis of GPx in erythrocytes demonstrates an overall reduced activity in children with ASD [18]. One recent study examined the activity of several GSH pathway enzymes in brain tissues from ten individuals with ASD and ten age-matched controls [24]. The enzymes examined included GST, GPx, GR and glutamate cysteine ligase (GCL), the rate-limiting enzyme for GSH synthesis. The activity of GPx, GST and GCL, but not GR, were found to be reduced in brain tissue of ASD compared with control individuals. Interestingly, by examining the catalytic and modulatory subunits of

GCL, these researchers noted that the GCL was not well modulated in ASD individuals. As an NADPH deficiency has been documented in ASD, the recycling of GSH from GSSG could be diminished since GR is an NADPH-dependent enzyme [25].

The reason for abnormalities in GSH enzyme activity is not clear. Polymorphisms in GSH pathway enzymes have undergone limited investigation in ASD. One study demonstrated transmission disequilibrium in the six alanine residue polyalanine repeat polymorphism of the GPx gene such that children with ASD were less likely to have this protective allele [26]. ASD has been associated with the GST M1 null genotype in one study [27] and in the context of the reduced folate carrier (RFC) 80 A>G genotype in another study [8]. One interesting study examined 308 single-nucleotide polymorphisms in 42 genes involved in the GSH pathway from 318 families in the Autism Genetic Resource Exchange [28]. The study found that ASD was associated with polymorphisms in glutaredoxin and cystathione γ lyase genes, as well as nominal marginal associations with the γ -glutamylcysteine synthetase catalytic subunit and cystathione γ lyase genes. These latter two genes are essential for the production of cysteine, the rate-limiting step for producing GSH. Factors other than genetics, such as nutritional factors, could also influence the activity of GSH enzymes. For example, GST is a selenium-dependent enzyme and selenium deficiency has been associated with ASD [29].

Abnormalities in other redox enzymes

Abnormalities in other redox enzymes have also been investigated in ASD. Superoxide, one of the major ROS produced in the cell, is converted to hydrogen peroxide by the enzyme superoxide dismutase. Hydrogen peroxide is converted into water through a GSH-dependent mechanism using GPx, or using the enzymes catalase or peroxiredoxin. Erythrocyte and plasma superoxide dismutase and catalase activity has been inconsistently reported to be abnormal in individuals with ASD [18]. Only a few studies have reported any genetic abnormalities in ASD related to these enzymes. A study examining pedigrees of Ashkenazi Jewish schizophrenic and autistic probands suggested the involvement of the chromosomal regions containing cytosolic superoxide dismutase [30]. Another study found that mitochondrial superoxide dismutase and catalase were among several genes found to exhibit differential alternative splicing in ASD boys compared with controls [31]. Reduced superoxide dismutase activity could cause elevation in superoxide radical, which can be converted to the more potent hydroxyl radical via the Fenton reaction and lead to oxidative damage of proteins, lipid and nucleic acids, while reduced activity of catalase

could cause the elimination of hydrogen peroxide by alternative pathways such as the GSH-dependent pathway or peroxiredoxin. Indeed, one study demonstrated higher levels of thioredoxin and peroxiredoxin I and III enzymes, as well as greater activity of thioredoxin reductase in children with autism compared with controls, suggesting that this important system for regulating protein cysteine redox status and eliminating hydrogen peroxide was most likely upregulated to compensate for deficits in glutathione metabolism [32].

Toxins

GSH is essential for the elimination of xenobiotics and heavy-metal ions from the body through two pathways. GSH is converted to phytochelatin by phytochelatin synthase. Phytochelatin combines with heavy-metal ions to eliminate these toxins from the body. Through another pathway, GSH is enzymatically conjugated to xenobiotics by GST to promote their elimination from the body. Both of these processes consume GSH. Given that environmental factors, particularly exposure to toxins such as organophosphates and heavy metals, have been associated with ASD, it is likely that environmental exposures that deplete the GSH pool for regulation of oxidative stress could be an etiological mechanism contributing to ASD [33]. Indeed, biomarkers of mercury toxicity have been correlated with glutathione abnormalities and autism severity [34].

Folate pathway abnormalities

Several factors that affect folate metabolism and impair folate transport across the blood–brain barrier and into neurons have been associated with ASD. ASD has been associated with polymorphisms in methylene tetrahydrofolate reductase (MTHFR) [18], dihydrofolate reductase [35] and RFC [8]. Folate is primarily transported across the blood–brain barrier by a receptor-mediated system that utilizes folate receptor alpha (FR α) [36]. Autoantibodies that bind to FR α , greatly impairing its function, have been associated with ASD [37–44]. Recently Frye *et al.* [37] demonstrated a very high prevalence of the blocking (60%) and binding (44%) FR α s in ASD children in an uncontrolled study. Ramaekers *et al.* [45] have independently verified this very high prevalence of the FR α -blocking autoantibody in ASD in a controlled study. Both RFC and FR α are essential for transporting folate across the blood–brain barrier, while dihydrofolate reductase is essential for reducing folic acid (the synthetic form of folate used to supplement food) into a form useable by the folate cycle. MTHFR is a major enzyme in the folate cycle that converts 5,10-methylene THF into 5-methyl THF, the substrate for methionine and SAM synthesis and the methionine methylation cycle. Thus,

the methyl groups transferred from SAM derive from 5-methylfolate via methionine synthase or betaine-homocysteinemethyl transferase (BHMT), and serve to link the folate cycle (one-carbon metabolism) with the methionine cycle (sulfur metabolism). Recent evidence has linked maternal folate insufficiency during pregnancy to an increased risk of offspring developing autism [46,47]. In post-pregnant mothers of children with autism, two independent studies have reported elevated homocysteine, decreased SAM levels and DNA hypomethylation [48,49]. These studies suggest that maternal folate status may be linked to increased risk of autism.

Metabolic abnormalities secondary to glutathione metabolism disturbances

Oxidative damage to cellular proteins, lipids & DNA

Oxidative damage to protein and nuclear DNA has been demonstrated in peripheral blood mononuclear cells and postmortem brains from ASD individuals [10,19]. In the brain, protein oxidation was found in cortical regions associated with speech, emotion and social behavior [50]. A recent controlled study reported increased oxidative damage to mitochondrial DNA in children with autism [51]. Although several studies have demonstrated abnormal lipid peroxidation in plasma, erythrocytes and urine in children with ASD, the results are inconsistent across studies [18,52].

Disrupted methylation metabolism

Methylation abnormalities have been documented in ASD children in four independent case–control studies from our laboratory [8,21,53,54], as well as the laboratory of others [55]. Most significantly, SAM and the SAM/SAH ratio, an index of methylation capacity, were significantly reduced in children with ASD. A reduced SAM/SAH ratio is known to be associated with hypomethylation of DNA, RNA, proteins and phospholipids, with functional consequences such as decreased gene and protein expression, enzyme activity and membrane phospholipid composition. The significant decrease in methylation capacity is consistent with studies from our laboratory, which demonstrate genome-wide DNA hypomethylation in primary leukocytes derived from children with ASD compared with paired sibling controls [54]. Importantly, the sibling control metabolic profile was not significantly different to age-matched neurotypical children, indicating that the abnormal profile was unique to autism, despite the similar environment and genetics between siblings. In children with autism, an increase in homocysteine is often accompanied by a decrease in SAM/SAH ratio and is associated with a decrease in cysteine, the rate-limiting amino acid for glutathione synthesis,

underscoring the connection between methylation capacity and glutathione synthesis.

Disruption in folate metabolism

Abnormalities of folate metabolism can disrupt methylation and result in genomic instability. An important product of the folate pathways is the *de novo* synthesis of purines, which are used as basic DNA building blocks and as precursors to several important metabolites. One of the purine products, guanosine, is the precursor to tetrahydrobiopterin (BH₄), an essential cofactor of several critical metabolic pathways, including the production of monoamine neurotransmitters, the breakdown of phenylalanine and the production of nitric oxide [6]. Abnormalities in several BH₄-related metabolic pathways have been associated with ASD, and cerebrospinal fluid BH₄ levels have been reported to be depressed in ASD [6,7]. Studies of genes coding for enzymes involved in BH₄ production have shown limited, inconsistent and complicated abnormalities related to ASD [56], suggesting that other factors, such as deficits in folate metabolism, could account for BH₄ deficits by limiting precursor availability.

Genetic alternations

Several studies have suggested that individuals with ASD have a general increase in chromosomal copy number variations, suggesting instability in chromosomal integrity [57]. In addition, studies have identified rare *de novo* mutations in ASD children not present in their parents, thereby pointing to acquired mutations and/or mutations secondary to errors in the maintenance of DNA integrity rather than inherited genetic syndromes [58,59]. Disturbances in both folate and methylation metabolism can lead to abnormalities in chromosomal integrity. Abnormalities in folate metabolism can limit the precursor pool of purines and pyrimidines required to replicate and repair DNA and folate deficiency has been shown to cause the expression of chromosomal fragile sites and chromosomal breakage [60]. In addition, DNA hypomethylation, which can occur because of abnormal methylation metabolism, can result in chromosomal instability [61].

Mitochondrial dysfunction

Mitochondria generate ROS and RNS as a byproduct of normal electron transport chain function, making adequate mitochondrial function dependent on adequate intramitochondria GSH concentrations. GSH cannot be synthesized in the mitochondria but is transported from the cytosol into the mitochondria through a special carrier. The synthesis of GSH requires ATP. Thus, mitochondrial dysfunction can reduce the ability of the

cell to produce new GSH, leaving the mitochondria more vulnerable to ROS/RNS.

Mitochondrial dysfunction is one of the most prevalent metabolic disorders affecting children with ASD [62,63]. The great majority of cases of mitochondrial disease in ASD have no associated mitochondrial or nuclear genetic abnormalities reported, suggesting that factors such as oxidative stress could be contributing to mitochondrial dysfunction [1,63]. Indeed, recent preliminary studies in our laboratory using immune cell lines and fresh immune cells demonstrate that a subset of children with ASD have mitochondria that are vulnerable to increased levels of oxidative stress. Our studies suggest that mitochondria in this subset of individuals with ASD appear to become overactive to compensate for chronic and acute increases in ROS [64]. These basic research findings parallel newly described mitochondrial disorders associated with ASD characterized by overactivity of complex I or IV [65,66]. In addition, recently Rose *et al.* demonstrated that a low GSH/GSSG redox ratio was correlated with suppression of redox-sensitive mitochondrial enzymes in the brain tissue of individuals with ASD [10].

Treatments developed using metabolic biomarkers

Currently the only well-accepted treatment for ASD symptoms is behavioral therapy that requires full-time engagement of a one-on-one therapist over several years. This therapy is not only expensive but is typically not covered by medical insurance, thereby limiting its application to a minority of children with ASD. Currently, the only US FDA medical treatments for ASD are antipsychotic medications that address associated, but not core, ASD symptoms. Several studies have developed treatments for ASD based on the biomarkers of abnormal GSH and associated metabolism. In this section we review these potentially disease-modifying treatments.

Folinic acid

Folinic acid is a reduced form of folate that can readily enter the folate cycle and can pass across the blood-brain barrier, despite the presence of the FR α autoantibody. Several cases series of children with ASD and cerebral folate deficiency, mostly caused by the FR α autoantibody, have documented significant gains, with some showing complete recovery with folinic acid treatment [38,39,41]. Using a prospective open-label clinical treatment protocol, we recently demonstrated that children with ASD and the FR α autoantibody exhibited significant improvements in receptive and expressive language, stereotyped behavior and attention with high-dose folinic acid (2 mg/kg/day; max: 50 mg/day) without significant adverse effects [37].

Methylcobalamin & folinic acid

A prospective, open-label study demonstrated that GSH metabolism could be normalized in children with ASD following a 3-month supplementation with subcutaneously injected methylcobalamin and oral folinic acid [53]. This treatment was associated with an average gain of 7.7 months in adaptive skills over the 3-month treatment period. Greater improvements in GSH metabolism were associated with a greater improvement in adaptive behavior [67].

Glutathione & glutathione precursors

One uncontrolled study demonstrated an increase in GSH during an 8-week open-label trial of oral lipopeptidic glutathione [68]. Another recent double-blind placebo-controlled study demonstrated that *N*-acetyl-L-cysteine improves irritability and social function in ASD children [69]. *N*-acetyl-L-cysteine provides the cysteine precursor to GSH and has been shown to increase intracellular GSH levels, although GSH levels were not measured in this study.

Antioxidant, multivitamin & mineral supplements

Several antioxidants [70], including vitamin C [71], carnosine [72] and L-carnitine [73,74], have been reported to significantly improve autistic behaviors, suggesting that treatment of oxidative stress could be beneficial for ASD children. Other studies suggest that glutathione metabolism can be improved by vitamins and minerals [25], as well as antioxidants, co-enzyme Q10 and B vitamin supplementation [75], and sapropterin treatment [4] in ASD children. Both small [69,73,74,76] and large [25] randomized controlled trials and large case series [4] demonstrate that novel treatments for children with ASD that can address oxidative stress are associated with improvements in core ASD symptoms [4,69,73,74], sleep and gastrointestinal symptoms [76], and hyperactivity, tantruming and parental impression of general functioning [25].

Sapropterin

The therapeutic effect of sapropterin – a synthetic form of BH₄ – on ASD symptoms has been reported in several clinical trials conducted over the past 25 years [6,77]. In an open-label study, sapropterin was found to improve redox metabolism leading to a fundamental alteration in BH₄ metabolism in ASD children [4].

Future perspective

In this article we have outlined the importance of redox regulation and GSH metabolism and associated pathways. We have outlined the primary abnormalities in GSH metabolism known to occur in children with ASD, as well as some of the potential abnormalities

associated with ASD that could result in abnormalities in the GSH pathway. In addition, we have outlined the potential consequences of abnormal GSH metabolism that are known to be associated with ASD and some of the ASD-related treatments that have been developed to target these metabolic abnormalities.

Some biomarkers of the metabolic pathways discussed have been consistently found to be abnormal across studies, such as low GSH and SAM, while other biomarkers have been inconsistently found to be abnormal. Future research will need to investigate which biomarkers are consistently abnormal and can be used to clinically determine a metabolic phenotype in ASD. Investigating the consistency of these biomarkers could assist in the understanding of whether there is a specific primary pathway that is abnormal that results in destabilization of other metabolic pathways, or whether weaknesses and deficits in several pathways are needed to metabolically destabilize these pathways. Such information would provide a sharper understanding of the metabolic phenotype(s) that are associated with ASD behaviors. Given the heterogeneity of autism, it is highly likely that different metabolic phenotypes will be associated with different subgroups of children. The clinical characterization of children who respond positively to a given intervention will be an important aspect of future research endeavors.

It is becoming clear that the etiology of most ASD cases involves complicated interactions between genetic predisposition with environmental exposures or triggers. The metabolic pathways discussed within this article demonstrate how multiple genes and environmental factors could come together to disrupt metabolism. However, a polygenic model with interactions between multiple genes and multiple environmental factors further complicates and confounds the search for candidate genes. For example, if an environmental trigger is a requisite factor, the same genetic profile could be present in unaffected individuals who did not receive the environmental insult during critical developmental windows. Future research should embrace these complicated models to further the understanding of underlying mechanisms that could lead to the development of ASD. Biomarkers discussed within this article should be particularly useful in understanding the connection between genetic predisposition and environmental triggers since biomarkers can reflect genetic polymorphisms that disrupt metabolic pathways as well as environmental exposures that interfere with normal metabolic flux. Most importantly, biomarkers are potentially useful for identifying those individuals who are most vulnerable to environmental triggers so they can be protected from developing pathology associated with autism.

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Executive summary**Autism spectrum disorder is associated with metabolic disturbances**

- Many children with autism spectrum disorder have disruptions in several physiological systems, including redox, folate, methylation (epigenetics) and mitochondrial metabolism, despite not having any defined genetic defect to account for these abnormalities.

Redox metabolism is important for maintaining cell health

- Redox metabolism balances reactive oxygen and nitrogen species to maintain a healthy redox potential in the cell.
- Abnormally high levels of reactive oxygen and nitrogen species can overwhelm the antioxidant mechanisms of the cell, resulting in oxidative stress, which can cause damage to the cellular molecules.

Glutathione metabolism is important for protecting the cell

- Glutathione metabolism is important for maintaining redox homeostasis and control of the levels of reactive oxygen and nitrogen species in the cell, and is important for detoxification and elimination of environmental toxins from the cell.

Glutathione metabolism abnormalities are associated with autism spectrum disorder

- Several studies have verified abnormalities in glutathione metabolism in plasma, immune cells and brains of children with autism spectrum disorder.

Glutathione metabolism is associated with abnormalities in other metabolic pathways in autism spectrum disorder

- Glutathione metabolism is associated with folate and methylation metabolism, both of which have abnormalities associated with autism spectrum disorder.
- The combination of glutathione metabolism abnormalities associated with abnormalities in folate and methylation pathways may represent a distinct endophenotype of autism.

Metabolic abnormalities may be amendable to treatment

- Studies have demonstrated that treatments that address metabolic abnormalities in the glutathione, methylation and folate pathways may improve both metabolic biomarkers and core symptoms of autism spectrum disorder.

References

- Rossignol DA, Frye RE. A review of research trends in physiological abnormalities in autism spectrum disorders: immune dysregulation, inflammation, oxidative stress, mitochondrial dysfunction and environmental toxicant exposures. *Mol. Psychiatry* 17(4), 389–401 (2012).
- Schaefer GB, Mendelsohn NJ. Genetics evaluation for the etiologic diagnosis of autism spectrum disorders. *Genet. Med.* 10(1), 4–12 (2008).
- Hallmayer J, Cleveland S, Torres A *et al.* Genetic heritability and shared environmental factors among twin pairs with autism. *Arch. Gen. Psychiatry* 68(11), 1095–1102 (2011).
- Frye RE, Delatorre R, Taylor HB *et al.* Metabolic effects of sapropterin treatment in autism spectrum disorder: a preliminary study. *Transl. Psychiatry* 3, e237 (2013).
- Rossignol D, Frye RE. Folate receptor alpha autoimmunity and cerebral folate deficiency in autism spectrum disorders. *J. Pediatr. Biochem.* 2, 263–271 (2012).
- Frye RE, Huffman LC, Elliott GR. Tetrahydrobiopterin as a novel therapeutic intervention for autism. *Neurotherapeutics* 7(3), 241–249 (2010).
- Frye RE. Central tetrahydrobiopterin concentration in neurodevelopmental disorders. *Front. Neurosci.* 4, 52 (2010).
- James SJ, Melnyk S, Jernigan S *et al.* Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 141B(8), 947–956 (2006).
- Evangelidou A, Vlachonikolis I, Mihailidou H *et al.* Application of a ketogenic diet in children with autistic behavior: pilot study. *J. Child Neurol.* 18(2), 113–118 (2003).
- Rose S, Melnyk S, Pavliv O *et al.* Evidence of oxidative damage and inflammation associated with low glutathione redox status in the autism brain. *Transl. Psychiatry* 2, e134 (2012).
- Sen CK. Cellular thiols and redox-regulated signal transduction. *Curr. Top. Cell. Regul.* 36, 1–30 (2000).
- Ristow M, Schmeisser S. Extending life span by increasing oxidative stress. *Free Radic. Biol. Med.* 51(2), 327–336 (2011).
- Milton VJ, Sweeney ST. Oxidative stress in synapse development and function. *Dev. Neurobiol.* 72(1), 100–110 (2012).
- Mcquillen PS, Ferriero DM. Selective vulnerability in the developing central nervous system. *Pediatr. Neurol.* 30(4), 227–235 (2004).

- 15 Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* 30(11), 1191–1212 (2001).
- 16 Vitvitsky V, Mosharov E, Tritt M, Ataullakhanov F, Banerjee R. Redox regulation of homocysteine-dependent glutathione synthesis. *Redox Rep.* 8(1), 57–63 (2003).
- 17 Banerjee RV, Matthews RG. Cobalamin-dependent methionine synthase. *FASEB J.* 4(5), 1450–1459 (1990).
- 18 Frustaci A, Neri M, Cesario A *et al.* Oxidative stress-related biomarkers in autism: systematic review and meta-analyses. *Free Radic. Biol. Med.* 52(10), 2128–2141 (2012).
- 19 Rose S, Melnyk S, Trusty TA *et al.* Intracellular and extracellular redox status and free radical generation in primary immune cells from children with autism. *Autism Res. Treat.* 986519 (2012).
- 20 James SJ, Rose S, Melnyk S *et al.* Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism. *FASEB J.* 23(8), 2374–2383 (2009).
- 21 James SJ, Cutler P, Melnyk S *et al.* Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. *Am. J. Clin. Nutr.* 80(6), 1611–1617 (2004).
- 22 James SJ, Melnyk S, Jernigan S *et al.* Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 141B(8), 947–956 (2006).
- 23 Geier DA, Kern JK, Garver CR, Adams JB, Audhya T, Geier MR. A prospective study of transsulfuration biomarkers in autistic disorders. *Neurochem. Res.* 34(2), 386–393 (2009).
- 24 Gu F, Chauhan V, Chauhan A. Impaired synthesis and antioxidant defense of glutathione in the cerebellum of autistic subjects: Alterations in the activities and protein expression of glutathione-related enzymes. *Free Radic. Biol. Med.* 65C, 488–496 (2013).
- 25 Adams JB, Audhya T, McDonough-Means S. Nutritional and metabolic status of children with autism vs. neurotypical children, and the association with autism severity. *Nutr. Metab.* 8, 34 (2011).
- 26 Ming X, Johnson WG, Stenroos ES, Mars A, Lambert GH, Buyske S. Genetic variant of glutathione peroxidase 1 in autism. *Brain Dev.* 32(2), 105–109 (2010).
- 27 Buyske S, Williams TA, Mars AE *et al.* Analysis of case–parent trios at a locus with a deletion allele: association of *GSTM1* with autism. *BMC Genet.* 7(1), 8 (2006).
- 28 Bowers K, Li Q, Bressler J, Avramopoulos D, Newschaffer C, Fallin MD. Glutathione pathway gene variation and risk of autism spectrum disorders. *J. Neurodev. Disord.* 3(2), 132–143 (2011).
- 29 Lakshmi Priya MD, Geetha A. Level of trace elements (copper, zinc, magnesium and selenium) and toxic elements (lead and mercury) in the hair and nail of children with autism. *Biol. Trace Elem. Res.* 142(2), 148–158 (2011).
- 30 Goodman AB. A family history study of schizophrenia spectrum disorders suggests new candidate genes in schizophrenia and autism. *Psychiatr. Q.* 65(4), 287–297 (1994).
- 31 Stamova BS, Tian Y, Nordahl CW *et al.* Evidence for differential alternative splicing in blood of young boys with autism spectrum disorders. *Mol. Autism* 4(1), 30 (2013).
- 32 Al-Yafee YA, Al-Ayadhi LY, Haq SH, El-Ansary AK. Novel metabolic biomarkers related to sulfur-dependent detoxification pathways in autistic patients of Saudi Arabia. *BMC Neurol.* 11, 139 (2011).
- 33 Herbert MR. Contributions of the environment and environmentally vulnerable physiology to autism spectrum disorders. *Curr. Opin. Neurol.* 23(2), 103–110 (2010).
- 34 Geier DA, Kern JK, Garver CR *et al.* Biomarkers of environmental toxicity and susceptibility in autism. *J. Neurol. Sci.* 280(1–2), 101–108 (2009).
- 35 Adams M, Lucock M, Stuart J, Fardell S, Baker K, Ng X. Preliminary evidence for involvement of the folate gene polymorphism 19bp deletion-DHFR in occurrence of autism. *Neurosci. Lett.* 422(1), 24–29 (2007).
- 36 Wollack JB, Makori B, Ahlawat S *et al.* Characterization of folate uptake by choroid plexus epithelial cells in a rat primary culture model. *J. Neurochem.* 104(6), 1494–1503 (2008).
- 37 Frye RE, Sequeira JM, Quadros EV, James SJ, Rossignol DA. Cerebral folate receptor autoantibodies in autism spectrum disorder. *Mol. Psychiatry* 18(3), 369–381 (2013).
- 38 Moretti P, Peters SU, Del Gaudio D *et al.* Brief report: autistic symptoms, developmental regression, mental retardation, epilepsy, and dyskinesias in CNS folate deficiency. *J. Autism Dev. Disord.* 38(6), 1170–1177 (2008).
- 39 Ramaekers VT, Blau N, Sequeira JM, Nassogne MC, Quadros EV. Folate receptor autoimmunity and cerebral folate deficiency in low-functioning autism with neurological deficits. *Neuropediatrics* 38(6), 276–281 (2007).
- 40 Ramaekers VT, Sequeira JM, Blau N, Quadros EV. A milk-free diet downregulates folate receptor autoimmunity in cerebral folate deficiency syndrome. *Dev. Med. Child Neurol.* 50(5), 346–352 (2008).
- 41 Ramaekers VT, Rothenberg SP, Sequeira JM *et al.* Autoantibodies to folate receptors in the cerebral folate deficiency syndrome. *N. Engl. J. Med.* 352(19), 1985–1991 (2005).
- 42 Ramaekers VT, Blau N. Cerebral folate deficiency. *Dev. Med. Child Neurol.* 46(12), 843–851 (2004).
- 43 Moretti P, Sahoo T, Hyland K *et al.* Cerebral folate deficiency with developmental delay, autism, and response to folic acid. *Neurology* 64(6), 1088–1090 (2005).
- 44 Shoffner J, Hyams L, Langley GN *et al.* Fever plus mitochondrial disease could be risk factors for autistic regression. *J. Child Neurol.* 25(4), 429–434 (2010).
- 45 Ramaekers VT, Quadros EV, Sequeira JM. Role of folate receptor autoantibodies in infantile autism. *Mol. Psychiatry* 18(3), 270–271 (2013).
- 46 Suren P, Roth C, Bresnahan M *et al.* Association between maternal use of folic acid supplements and risk of autism spectrum disorders in children. *JAMA* 309(6), 570–577 (2013).

- 47 Schmidt RJ, Tancredi DJ, Ozonoff S *et al.* Maternal periconceptional folic acid intake and risk of autism spectrum disorders and developmental delay in the CHARGE (CHildhood Autism Risks from Genetics and Environment) case-control study. *Am. J. Clin. Nutr.* 96(1), 80–89 (2012).
- 48 James SJ, Melnyk S, Jernigan S, Hubanks A, Rose S, Gaylor DW. Abnormal transmethylation/transsulfuration metabolism and DNA hypomethylation among parents of children with autism. *J. Autism Dev. Disord.* 38(10), 1966–1975 (2008).
- 49 James SJ, Melnyk S, Jernigan S *et al.* A functional polymorphism in the reduced folate carrier gene and DNA hypomethylation in mothers of children with autism. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 153b(6), 1209–1220 (2010).
- 50 Sajdel-Sulkowska EM, Xu M, McGinnis W, Koibuchi N. Brain region-specific changes in oxidative stress and neurotrophin levels in autism spectrum disorders (ASD). *Cerebellum* 10(1), 43–48 (2011).
- 51 Napoli E, Wong S, Giulivi C. Evidence of reactive oxygen species-mediated damage to mitochondrial DNA in children with typical autism. *Mol. Autism* 4(1), 2 (2013).
- 52 Damodaran LP, Arumugam G. Urinary oxidative stress markers in children with autism. *Redox Rep.* 16(5), 216–222 (2011).
- 53 James SJ, Melnyk S, Fuchs G *et al.* Efficacy of methylcobalamin and folinic acid treatment on glutathione redox status in children with autism. *Am. J. Clin. Nutr.* 89(1), 425–430 (2009).
- 54 Melnyk S, Fuchs GJ, Schulz E *et al.* Metabolic imbalance associated with methylation dysregulation and oxidative damage in children with autism. *J. Autism Dev. Disord.* 42(3), 367–377 (2012).
- 55 Suh JH, Walsh WJ, McGinnis WR, Lewis A, Ames BN. Altered sulfur amino acid metabolism in immune cells of children diagnosed with autism. *Am. J. Biochem. Biotechnol.* 4(2), 105–113 (2008).
- 56 Schnetz-Boutaud NC, Anderson BM, Brown KD *et al.* Examination of tetrahydrobiopterin pathway genes in autism. *Genes Brain Behav.* 8(8), 753–757 (2009).
- 57 Devlin B, Scherer SW. Genetic architecture in autism spectrum disorder. *Curr. Opin. Genet. Dev.* 22(3), 229–237 (2012).
- 58 Neale BM, Kou Y, Liu L *et al.* Patterns and rates of exonic *de novo* mutations in autism spectrum disorders. *Nature* 485(7397), 242–245 (2012).
- 59 Sanders SJ, Murtha MT, Gupta AR *et al.* *De novo* mutations revealed by whole-exome sequencing are strongly associated with autism. *Nature* 485(7397), 237–241 (2012).
- 60 Fenech M. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. *Mutat. Res.* 733(1–2), 21–33 (2012).
- 61 Nishida N, Kudo M, Nishimura T *et al.* Unique association between global DNA hypomethylation and chromosomal alterations in human hepatocellular carcinoma. *PLoS ONE* 8(9), e72312 (2013).
- 62 Frye RE, Rossignol DA. Mitochondrial dysfunction can connect the diverse medical symptoms associated with autism spectrum disorders. *Pediatr. Res.* 69(5 Pt 2), 41R–47R (2011).
- 63 Rossignol DA, Frye RE. Mitochondrial dysfunction in autism spectrum disorders: a systematic review and meta-analysis. *Mol. Psychiatry* 17(3), 290–314 (2012).
- 64 Rose S, Frye RE, Slattery J *et al.* Oxidative stress induces mitochondrial dysfunction in a subset of autism lymphoblastoid cell lines in a well-matched case control cohort. *PLoS ONE* 9(1), e85436 (2014).
- 65 Frye RE, Naviaux RK. Autistic disorder with complex IV overactivity: a new mitochondrial syndrome. *J. Pediatr. Neurol.* 9, 427–434 (2011).
- 66 Graf WD, Marin-Garcia J, Gao HG *et al.* Autism associated with the mitochondrial DNA G8363A transfer RNA(Lys) mutation. *J. Child Neurol.* 15(6), 357–361 (2000).
- 67 Frye RE, Melnyk S, Fuchs G *et al.* Effectiveness of methylcobalamin and folinic acid treatment on adaptive behavior in children with autistic disorder is related to glutathione redox status. *Autism Res. Treat.* 609705 (2013).
- 68 Kern JK, Geier DA, Adams JB, Garver CR, Audhya T, Geier MR. A clinical trial of glutathione supplementation in autism spectrum disorders. *Med. Sci. Monit.* 17(12), CR677–CR682 (2011).
- 69 Hardan AY, Fung LK, Libove RA *et al.* A randomized controlled pilot trial of oral *N*-acetylcysteine in children with autism. *Biol. Psychiatry* 71(11), 956–961 (2012).
- 70 Rossignol DA. Novel and emerging treatments for autism spectrum disorders: a systematic review. *Ann. Clin. Psychiatry* 21(4), 213–236 (2009).
- 71 Dolske MC, Spollen J, Mckay S, Lancashire E, Tolbert L. A preliminary trial of ascorbic acid as supplemental therapy for autism. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 17(5), 765–774 (1993).
- 72 Chez MG, Buchanan CP, Aimonovitch MC *et al.* Double-blind, placebo-controlled study of L-carnosine supplementation in children with autistic spectrum disorders. *J. Child Neurol.* 17(11), 833–837 (2002).
- 73 Geier DA, Kern JK, Davis G *et al.* A prospective double-blind, randomized clinical trial of levocarnitine to treat autism spectrum disorders. *Med. Sci. Monit.* 17(6), PI15–PI23 (2011).
- 74 Fahmy SF, El-Hamamsy MH, Zaki OK, Badary OA. L-carnitine supplementation improves the behavioral symptoms in autistic children. *Res. Autism Spectr. Disord.* 7, 159–166 (2013).
- 75 Frye RE, Delatorre R, Taylor H *et al.* Redox metabolism abnormalities in autistic children associated with mitochondrial disease. *Transl. Psychiatry* 3, e273 (2013).
- 76 Adams JB, Holloway C. Pilot study of a moderate dose multivitamin/mineral supplement for children with autistic spectrum disorder. *J. Altern. Complement. Med.* 10(6), 1033–1039 (2004).
- 77 Klaiman C, Huffman L, Masaki L, Elliott GR. Tetrahydrobiopterin as a treatment for autism spectrum disorders: a double-blind, placebo-controlled trial. *J. Child Adolesc. Psychopharmacol.* 23(5), 320–328 (2013).