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-
oxidative stress is induced. When high levels of ROS/RNS are 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 comprised 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. 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. 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; M5: Methionine synthase; MTHFR: Methylene tetrahydrofolate reductase; SAM: S-adenosyl methionine; SAH: S-adenosylhomocysteine; THF: Tetrahydrofolate.
Abnormalities in glutathione metabolism and 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].

Abnormalities in glutathione metabolism associated with autism spectrum disorder

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 abnormal in individuals with 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 cystathionine γ lyase genes, as well as nominal marginal associations with the γ-glutamylcysteine synthetase catalytic subunit and cystathionine 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 found 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 phytochelatine by phytochelatine synthase. Phytochelatine 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 etiologic 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 (FRα) [36]. Autoantibodies that bind to FRα, 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%) FRαs in ASD children in an uncontrolled study. Ramaekers et al. [45] have independently verified this very high prevalence of the FRα-blocking autoantibody in ASD in a controlled study. Both RFC and FRα 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.

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 (BH$_4$), 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 BH$_4$-related metabolic pathways have been associated with ASD, and cerebrospinal fluid BH$_4$ levels have been reported to be depressed in ASD [6,7]. Studies of genes coding for enzymes involved in BH$_4$ 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 BH$_4$ 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 that 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 replace 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 FRα autoantibody. Several cases series of children with ASD and cerebral folate deficiency, mostly caused by the FRα 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 FRα 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 lipoceutical 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


