



# Abnormal Porphyrin Metabolism in Autism Spectrum Disorder and Therapeutic Implications

Neluwa-Liyanage R. Indika<sup>1</sup> · Udara D. Senarathne<sup>1,2</sup> · Antonio Malvaso<sup>3</sup> · Dhanushka Darshana<sup>4</sup> · Susan C. Owens<sup>5</sup> · Borhan Mansouri<sup>6</sup> · Yuliya Semenova<sup>7</sup> · Geir Bjørklund<sup>8</sup>

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## Abstract

Autism spectrum disorder (ASD) is a mosaic of neurodevelopmental conditions composed of early-onset social interaction and communication deficits, along with repetitive and/or restricted patterns of activities, behavior, and interests. ASD affects around 1% of children worldwide, with a male predominance. Energy, porphyrin, and neurotransmitter homeostasis are the key metabolic pathways affected by heavy metal exposure, potentially implicated in the pathogenesis of ASD. Exposure to heavy metals can lead to an altered porphyrin metabolism due to enzyme inhibition by heavy metals. Heavy metal exposure, inborn genetic susceptibility, and abnormal thiol and selenol metabolism may play a significant role in the urinary porphyrin profile anomalies observed in ASD. Altered porphyrin metabolism in ASD may also be associated with, vitamin B6 deficiency, hyperoxalemia, hyperhomocysteinemia, and hypomagnesemia. The present review considers the abnormal porphyrin metabolism in ASD in relation to the potential pathogenic mechanism and discusses the possible metabolic therapies such as vitamins, minerals, cofactors, and antioxidants that need to be explored in future research. Such targeted therapeutic therapies would bring about favorable outcomes such as improvements in core and co-occurring symptoms.

**Keywords** Autism · Porphyrin · Mercury · Lead · Heavy metal · Chelator · Thiol

✉ Neluwa-Liyanage R. Indika  
ind.liyanage@sjp.ac.lk

✉ Geir Bjørklund  
bjorklund@conem.org

- 1 Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Nugegoda 10250, Sri Lanka
- 2 Department of Chemical Pathology, Monash Health Pathology, Monash Health, Clayton, Victoria, Australia
- 3 IRCCS “C. Mondino” Foundation, National Neurological Institute, Department of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy
- 4 Department of Pharmacy, Faculty of Allied Health Sciences, University of Ruhuna, Galle, Sri Lanka
- 5 Autism Oxalate Project, Autism Research Institute, San Diego, CA, USA
- 6 Substance Abuse Prevention Research Center, Research Institute for Health, Kermanshah University of Medical Sciences, Kermanshah, Iran
- 7 Nazarbayev University School of Medicine, Astana, Kazakhstan
- 8 Council for Nutritional and Environmental Medicine, Toften 24, 8610 Mo i Rana, Norway

## Abbreviations

AGT	Alanine-glyoxylate aminotransferase
AIP	Acute intermittent porphyria
ALA	Delta-aminolevulinic acid
ALAD	Delta-aminolevulinic acid dehydratase
ALAS1	Delta-aminolevulinic acid synthase 1
AS	Asperger’s syndrome
ASD	Autism spectrum disorder
ATEC	Autism Treatment Evaluation Checklist
CBS	Cystathionine beta-synthase
Cd	Cadmium
CGL	Cystathionine-gamma lyase
CPOX	Coproporphyrinogen III oxidase
DHLA	Dihydrolipoic acid
DMPS	Dimercaptopropane-1-sulfonic acid
DMSA	Dimercaptosuccinic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
EDTA	Ethylenediaminetetraacetic acid
Hg	Mercury
Mg	Magnesium
PBGS	Porphobilinogen synthase
Pb	Lead

TMP	Thiamine monophosphate
TPP	Thiamine pyrophosphate
TTFD	Thiamine tetrahydrofurfuryl disulfide
UROD	Uroporphyrin decarboxylase
Zn	Zinc

## Introduction

Autism spectrum disorder (ASD) is a mosaic of neurodevelopmental disorder composed of early-onset deficits of social interaction and communication, as well as repetitive and/or restricted patterns of activities, behavior, and interests associated with a number of genetic and environmental risk factors [1]. It is a spectrum disorder with significant heterogeneity in nature, severity, and progression of core symptoms, cognitive development, language abilities, and other co-occurring problems [2]. According to recent consensus, ASD affects around 1% of children worldwide, with males having a higher prevalence [3–5]. Furthermore, as stated by Zeidan et al., ASD prevalence has expanded over time, varying substantially across different socio-demographics [3]. According to the latest estimates in 2020 from Autism and Developmental Disabilities Monitoring (ADDM) Network, about 1 in 36 children in the USA are identified with ASD, which is a significant increase compared to the estimates of 1 in 150 children in 2000.

With no reliable molecular biomarkers to guide the diagnosis, ASD is diagnosed based on clinical criteria defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) [6]. The DSM-5 recognizes that ASD can coexist with other disorders [6], including sleep disorders [7–9]; epilepsy [10]; other neurodevelopmental, mental, or behavioral disorders [1]; inherited metabolic disorders [11]; and other genetic syndromes [12, 13]. Accordingly, the etiology of ASD is multifactorial and involves a spectrum of genetic, epigenetic, and environmental factors [14]. Moreover, a plethora of research on molecular biomarker studies exploring genetics, metabolic biomarkers, autoantibodies, and cytokine profile in ASD has shown that the symptoms are associated with several dysfunctions including intestinal dysbiosis [15], immune and metabolic abnormalities, mitochondrial dysfunction, environmental toxicant exposures, and oxidative stress [16]. Interplay in these dysfunctions may lead to abnormalities in early brain development with altered brain connectivity, exaggerated synaptic pruning, imbalance of excitatory and inhibitory neurotransmitters, and other neuronal-level abnormalities leading to ASD [17].

The key metabolic pathways potentially implicated in the pathogenesis of ASD include energy [18, 19], amino acid [20, 21], serotonin [22], dopamine [23], one-carbon [24–26], purine [27], and porphyrin metabolism [28–31]. Porphyrins are a group of naturally occurring compounds

with great importance in the metabolic processes of many organisms including humans. Various porphyrin derivatives are biosynthesized from  $\delta$ -aminolevulinic acid and glycine during human haem biosynthesis. The core structure of porphyrin is called porphine, and different substitutions on the porphine ring result in porphyrin derivatives such as uroporphyrin, coproporphyrin, and protoporphyrin [32]. Defects in the haem biosynthetic pathway due to genetic and/or environmental factors lead to the accumulation of metabolic intermediates and abnormal porphyrin excretion (porphyrinuria) [33].

Porphyria in ASD has often been attributed to heavy metal toxicity [34–40]. However, the heterogeneity of the underlying factors involved in ASD has prompted researchers to reconsider their attempts to comprehend the neurochemical changes underlying ASD, including porphyrin metabolism. Therefore, it may be anecdotal to attribute abnormal porphyrin excretion profile to heavy metal toxicity alone. The novel research findings concerning neurochemical abnormalities in autism etiology propose possible other pathogenic mechanisms with implications on alternative diagnostic and therapeutic approaches that could enhance the quality of ASD interventions. The present review considers the abnormal porphyrin metabolism in ASD in relation to plausible alternative biochemical mechanisms and discusses the possible therapeutic implications.

## Porphyrin Metabolites in ASD Subjects

Urinary porphyrin levels in ASD have been explored by several study groups. However, most of them were conducted by a few research teams in the USA [28, 29, 31, 36–38, 41, 42], while the rest were conducted in Australia [43, 44], France [41], South Korea [45], Egypt [34] Slovenia [35], and Armenia [46].

Evaluation of any urinary metabolite requires normalization to a ubiquitous urinary metabolite such as creatinine. Although it was once hypothesized a possible correlation between ASD and depressed urinary creatinine levels by Whiteley et al., 2 years later, Nataf et al. concluded their study on porphyria in childhood ASD, refuting the former finding further stating that urinary porphyrin levels were elevated in ASD individuals when normalized to creatinine [30].

When normalized to creatinine, coproporphyrin levels [29, 31, 34, 36, 41], precoproporphyrin [31, 34, 36, 41, 45], pentacarboxyporphyrin [29, 31, 34, 41, 45], and hexacarboxyporphyrin [31, 34, 41] levels have been found to be higher in ASD group compared to controls, while heptacarboxyporphyrin and uroporphyrin have been reported to be higher in ASD group only in a single study [34]. It is important to note that even when the reported differences of

specific porphyrins are not statistically significant, the trend was towards higher levels in ASD compared to controls.

Uroporphyrin levels have not been reported to be significantly different from the controls except in a single study [34] and considered a marker independent of environmental toxicity [41]. Therefore, several study groups have used specific porphyrin to uroporphyrin ratios to eliminate the effects of variations in urinary volume. Coproporphyrin/uroporphyrin ratio and precoproporphyrin/uroporphyrin ratio have been reported to be higher in ASD group [37, 41]. Studies reporting specific porphyrin levels normalized to creatinine and uroporphyrin levels are summarized in Table 1.

Geier et al. and Khaled et al. also compared porphyrin levels between subjects with ASD and controls without normalizing to creatinine in several publications [28, 34, 37, 38]. However, these findings also showed increased pentacarboxyporphyrin and coproporphyrin levels in children with ASD.

Woods et al. reported that higher porphyrin concentrations were found in young children, particularly uro-, hepta-, and coproporphyrins, although their concentrations were observed to decline with age, possibly due to age-related differences in haem biosynthesis [31]. This indicates that in analyzing the differences of porphyrin levels between cases and controls, the moderating effects of age should be controlled by using such statistical models as ANCOVA.

## Urinary Porphyrin as a Potential Biomarker of Heavy Metal Exposure

Many researchers have investigated the correlation between ASD, abnormal urinary porphyrin profile, and the possible causal impact of heavy metals on the correlation [41, 47, 48]. For instance, Nataf et al. demonstrated that average elevation of coproporphyrin levels in autistic disorder was comparable to the elevation observed in subjects with significant arsenic (As) and mercury (Hg) exposure [41]. This speculation is further strengthened by demonstrating a reduction in urinary porphyrins following the treatment of ASD children or rats exposed to methylmercury hydroxide, with dimercaptosuccinic acid (DMSA/succimer), a heavy metal chelator [30, 37, 39, 49, 50].

Many study groups proposed that the abnormally high porphyrin levels in subjects with ASD could be due to Hg toxicity. For instance, precoproporphyrin, an atypical porphyrin, previously described primarily in animals and humans exposed to Hg or Hg compounds, was found in higher concentrations in the urine of children with ASD [31, 41]. Additionally, the differences in Hg toxicity-associated porphyrin levels from different geographical areas suggest the interplay of variations in the environmental factors and genetic predispositions in detoxification capacity [51].

The biosynthesis of haem begins with delta-aminolevulinic acid (ALA) formation and ends with the insertion of iron into the protoporphyrin IX ring [52]. Exposure to heavy metals such as Pb and Hg can lead to several metabolic disturbances, with heavy metals being known direct or indirect inhibitors of several metabolic pathways in the human body.

The meta-analysis by Zhang et al. revealed higher levels of Hg and Pb in children with ASD compared to controls [53]. Another study demonstrated that following chelation therapy with DMSA, urinary Hg concentrations increased from three to approximately five folds higher in ASD subjects compared to healthy controls, while Pb and cadmium (Cd) levels in urine showed only statistically non-significant increase [54]. Windham et al. suggested a possible association between ASD and estimated environmental exposure of potential neuro- and developmental toxicants near the birth region in their 2006 case-control study, and seven more ecological studies revealed a correlation between environmental Hg and ASD, while another study uncovered a similar association to Pb [55–63]. Some studies, however, observed no statistically significant difference in urinary Hg levels or previous Hg exposure as measured by fish consumption, vaccines received, or the number of dental amalgam fillings in subjects with ASD compared to the controls [31]. Furthermore, Adams et al. demonstrated a significantly strong correlation between toxic metal excretion and behavioral measures of ASD [48].

In contrast to Pb and Hg, meta-analysis of zinc (Zn) levels in blood showed significantly lower levels in subjects with ASD compared to neurotypical controls [53, 64]. Atypical porphyrin levels might be a consequence of the imbalance between the level of heavy metal exposure and protective factors such as detoxification capacity and sufficiency of Zn at least in a subset of ASD individuals. For instance, a young ASD subject with low-level Hg exposure might have relatively higher urine atypical porphyrins in the presence of low detoxification capacity. Furthermore, Zn deficiency may potentiate the toxic effects of heavy metals even at relatively normal levels [65].

The main target in Pb poisoning is delta-aminolevulinic acid dehydratase (ALAD), also known as porphobilinogen synthase (PBGs). The main mechanism of inhibition is the oxidation of sulfhydryl groups located at the active site of ALAD [66]. The lone electron pair on Pb(II) could also sterically interfere with the substrate or product binding to ALAD enzyme [67]. The inhibition of ALAD causes the accumulation of ALA. As cited by Akshatha et al., ALA which is implicated in symptoms of porphyria alters neurotransmitter metabolism, induces mitochondrial dysfunction, inhibits melatonin release, and increases production of reactive oxygen species, which are also relevant in pathogenesis of ASD [68]. Interestingly, there are case reports describing

**Table 1** Porphyrin profile in autism spectrum disorder (ASD) and Asperger's syndrome (AS) in comparison to neurotypical controls (NTCs) or sibling controls (SCs). Adapted from studies reporting porphyrin levels normalized to creatinine and urine porphyrin ratios. *AD-F* females with autistic disorder, *AD-M* males autistic disorder, with AS Asperger syndrome, *ASD* autism spectrum disorder, *cP* coproporphyrin, *Cr* creatinine, *7cxP* heptacarboxyporphyrin, *6cxP* hexacarboxyporphyrin, *NR* not reported, *NTCs* neurotypical controls, *NTCs-F* female neurotypical controls, *NTCs-M* male neurotypical controls, *5cxP* pentacarboxyporphyrin, *PDD-NOS* pervasive developmental disorder-not otherwise specified, *pre-cP* precoproporphyrin, *RV* reference values, *SCs* sibling controls, *tP* total porphyrins, *uP* uroporphyrin. \**p* ≤ 0.05; \*\**p* ≤ 0.01; \*\*\**p* ≤ 0.001; †, ANOVA *F*-test; †, significantly increased in case group; →, statistically insignificant difference

Study (country)	Mean age (age range) in years	Group (N)	uP/Cr	7cxP/Cr	7cxP/uP	6cxP/Cr	5cxP/Cr	preP/Cr	pre-cP/uP	cP/Cr	cP/uP	cP I/Cr	cP III/Cr	tP/Cr
Nataf et al. (2006) (France)	AD: 6.4	AS (11) vs. NTCs (12)	→	→	NR	→	→	→	→	→	→	NR	NR	NR
	AS: 10	AD (106) vs. NTCs (12)	→	→	NR	→	→	→	→	→	→	NR	NR	NR
	PDD: 6.6	PDD-NOS (66) vs. NTCs (12)	→	→	NR	→	→	→	→	→	→	NR	NR	NR
Geier and Geier (2007) (USA)	ASD: (NR)	ASD (11) vs. NTCs (5)	NR	NR	→	NR	NR	NR	→	NR	→	NR	NR	NR
	NTCs: (20-59)	ASD (20) vs. NTCs (20)	→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
Kern et al. (2011b) (USA)	ASD: 6.6 (2-13)	AD (30) vs. NTCs (32)	NR	NR	NR	→	→	NR	NR	→	NR	NR	NR	NR
	NTC: 6.75 (3-13)	PDD-NOS (14) vs. NTCs (32)	NR	NR	NR	→	→	NR	NR	→	NR	NR	NR	NR
Heyer et al. (2012) (USA)	AD: 6.08 (3.2-11.3)	†AD-M (59) vs. NTCs-M (59)	→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	PDD: 6.59 (2.5-12.4)	†AD-F (55) vs. NTCs-F (5)	→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
Woods et al. (2010) (USA)	NTCs: 7.18 (2.3-11.7)	ASD (65) vs. NTCs (9)	→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	AD-M: 6.01	AD (40) vs. NTCs (40)	→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
Youn et al. (2010) (South Korea)	AD-F: 4.60	AD (40) vs. SCs (20)	→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	NTCs-M: 6.39	ASD (12) vs. RV	→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
Khaled et al. (2016) (Egypt)	NTCs-F: 6.93		→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	ASD: 6.7 (2-6)		→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
Harutyunyan et al. (2021) (Armenia)	NTCs: 4.6 (2-16)		→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	AD: 4.118 (3-6)		→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	NTCs: 5.23 (3-6)		→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	SCs: 9.5 (3-15)		→	→	NR	→	→	→	NR	→	NR	NR	NR	NR
	ASD: (3-6)		→	→	NR	→	→	→	NR	→	NR	NR	NR	NR

patients with Pb toxicity presenting with clinical features of porphyria and ASD [69].

Coproporphyrinogen III oxidase (CPOX) seems to be the second vulnerable enzyme, catalyzing the two-step decarboxylation of coproporphyrinogen III into protoporphyrinogen IX [52]. Impaired CPOX activity leads to increased coproporphyrin levels in urine. Pb does not exert any direct inhibitory effect by its binding to the active site of the enzyme. As the enzyme is located in the mitochondrial intermembrane space, Pb-induced mitochondrial membrane distortion has been discussed as a potential culprit [70]. However, Hg binding to thiol groups of the CPOX enzyme (e.g., C319 cysteine moiety) could interfere with the structure and directly inhibit the conversion of harderoporphyrinogen to protoporphyrinogen [71].

Uroporphyrin decarboxylase (UROD) is also inhibited by Hg binding to thiol groups. Accordingly, the inhibition can be prevented by the thiols [72]. While Hg and Cd may inhibit ferrochelatase directly, Pb is thought to reduce iron availability for  $\text{Fe}^{2+}$  ferrochelatase activity in the cell, leading to Zn protoporphyrin accumulation owing to  $\text{Zn}^{2+}$  ferrochelatase activity unopposed by  $\text{Fe}^{2+}$  [70].

*ALAD* gene exhibits polymorphisms due to transversion of the base at position 177, producing two codominant alleles, namely *ALAD1* and *ALAD2*. Consequently, three genotypes are formed by pairs of alleles: *ALAD1-1*, *ALAD1-2*, and *ALAD2-2*. *ALAD2* protein binds Pb more tightly to the enzyme than *ALAD1* protein due to the substitution of asparagine for lysine at the 59th position, resulting in more effective inactivation of *ALAD* and elevated Pb concentration in the body [73]. Therefore, it has been hypothesized that *ALAD-2* may reduce harmful effects by conferring a protective effect by sequestering circulating Pb, thus preventing toxic effects [74]. However, recent evidence suggests that the *ALAD1-2* genotype may be associated with a higher risk of long-term Pb toxicity than the *ALAD1-1* genotype. The *ALAD1-2* heterozygosity was significantly more common in autistic children than in controls, indicating that children with the *ALAD2* variant were approximately 1.67 times more likely to be autistic [75]. Intriguingly, the maternal *ALAD2* variant was associated with higher placental Pb levels [76]. The effects of higher placental Pb levels related to the *ALAD2* variant on the fetus are yet to be explored.

In human subjects with low-level Hg exposure, a specific variant (A814C) in exon 4 of the human *CPOX* gene (*CPOX4*) encoding an N272H substitution is linked with anomalous urinary porphyrin efflux and increased neurobehavioral deficits. Studies suggest that this variant encodes an enzyme with a lower affinity for coproporphyrinogen-III [71]. This gene product preferentially converts the upstream penta-carboxylporphyrin to ketoisocoporphyrin. Hg-exposed subjects carrying the *CPOX4* variant exhibit increased levels of penta-carboxylporphyrin due to

inhibition of the penta- to the tetra-decarboxylation step of UROD, with elevated excretion of tetra-carboxyl porphyrins and atypical ketoisocoporphyrin [77].

Taken together, characteristic changes in urine porphyrin levels of Hg toxicity include elevated levels of tetra- and penta-carboxylporphyrin and the appearance of an atypical metabolite pre-coproporphyrin [78]. Additionally, in subjects harboring *CPOX4*, atypical porphyrin ketoisocoporphyrin is also increased [77]. Pb toxicity can lead to the elevation of urine coproporphyrin [70, 79], erythrocyte Zn protoporphyrin, and urinary ALA without any increase in urine porphobilinogen [80, 81]. Interestingly, blood lead levels have been shown to negatively correlate with low serum Zn levels in school children [82].

### Can Heavy Metal-Induced Toxicity Explain Sex Differences in Autism Spectrum Disorder?

Meta-analysis of prevalence studies demonstrated a male-to-female ratio close to 3:1 in ASD [83]. A perfect pathogenic model proposed for ASD should be able to explain this male preponderance. This could be explained by the presence of sexual dimorphism in terms of higher body burdens of heavy metals or increased susceptibility to the effects of exposure. In addition, influences of gonadal hormones, epigenetic modifications, and sex chromosome activation may be involved in gender differences in exposure outcomes [84].

Heavy metals appear to exhibit gender-specific effects that could be explained by lesser glutathione availability, lesser sulfate-based detoxification capacity, potentiating effects of testosterone, and greater neuroinflammatory response in males [85]. According to large-scale epidemiological studies, males have greater mean blood Pb levels than females. However, the differences in blood Pb levels are not significant at birth or early in life. Surprisingly, the differences intensified around the age of 12 years, correlating with puberty. Furthermore, males appear to be more susceptible to the impact of prenatal and postnatal Pb exposure. Postnatal and early childhood exposure was associated with attention span and cognitive development with males being more adversely affected with lower cognitive function scores [84, 86].

Evidence supporting potential sexual dimorphism associated with prenatal and early postnatal exposure to Hg is sparse. The results show that sex differences in Hg metabolism are only noticeable at high Hg concentrations. Males seem more prone to deficits in motor functions and cognitive and neurobehavioral performance when exposed to Hg. Unlike Pb, however, most of these studies have been carried out in people exposed to relatively greater levels of Hg. Therefore, it is uncertain if the dimorphism in neurotoxicity

persists at lower exposure levels [86]. Furthermore, Hg exposure at the age of nine years was linked to poorer mental agility and worse neurobehavioral development, short-term memory, visual memory processes, and sequential organization in Spanish children. This association has been altered by gender and specific genetic variants in *BDNF*, *APOE*, and *GSTP1* [87].

Some studies reported evidence supporting the presence of sexual dimorphism in the glutathione system, although most studies indicated the lack of sex-specific differences [88]. A Jamaican study found that ASD patients with *GSTP1* Ile105Val genotype Ile/Ile correspond to higher blood Hg concentrations compared to genotype Ile/Val. *GSTP1* encodes a glutathione S-transferase enzyme that catalyzes the formation of glutathione conjugates. This finding suggests that defects in *GSTP1*-mediated detoxification of Hg play a potential role in the pathogenesis of autism [89]. Sexual dimorphism in the glutathione system appears to be affected by several confounding variables, including age and brain region [88].

## The Usefulness of Porphyrins as Biomarkers in ASD

Certain porphyrin metabolites appear as candidate biomarkers with relatively low sensitivity but nearly 100% specificity [29]. Moreover, the urine porphyrin levels positively correlated with the severity of autism [28, 42, 44]. For instance, pentacarboxyporphyrin, precoproporphyrin, and coproporphyrin levels are higher in severe cases with ASD compared to mild cases [28, 42]. Correspondingly, subjects with Asperger syndrome, who are usually mildly affected, with better language and cognitive skills did not show significant differences in urine porphyrin levels compared to the control group. Moreover, abnormal porphyrin levels were reported to be significantly higher in children with autistic disorder associated with epilepsy [41].

As the name implies, ASD is a spectrum disorder with significant heterogeneity of clinical features and underlying pathogenic mechanisms. This explains why the biomarkers yield less sensitivity and higher specificity. For instance, ASD subjects with a pathogenic mechanism apart from heavy metal toxicity or reduced capacity to detoxify heavy metals may yield negative test results for urine porphyrin assessment. The primary goal of such a test is to accurately identify a subset of individuals with shared pathogenicity, predict prognosis, and attempt metal-type-targeted therapy. Prospective follow-up studies are needed to evaluate the use of urine porphyrin biomarkers to predict prognosis.

Urine porphyrin levels appear to be a good biomarker to monitor response to therapy. The differences in urine

porphyrin biomarkers between chelated ASD subjects and non-chelated ASD subjects were statistically significant [37, 38]. Importantly, the test can be performed on an untimed spot urine sample, a convenient, non-invasive sampling strategy. This is particularly beneficial as children with ASD can be hypersensitive to venipuncture procedures. On the other hand, the test is relatively inexpensive.

## Shared Pathogenic Mechanisms in Porphyria and ASD

Despite the presence of porphyria in ASD as reported consistently by many study groups, genetic porphyrias co-occurring with ASD have not been common with only a single case report of an ASD patient with acute intermittent porphyria (AIP) reported in the online literature [90]. However, porphyria is a commonly underdiagnosed entity due to non-specific symptoms [68].

There are striking similarities between the symptomatology of porphyria and ASD such as abdominal pain [91, 92], vomiting [91, 92], constipation [91, 92], seizures [10, 91, 93], and behavioral changes [91, 94, 95]. Even more striking are the biochemical similarities; porphyrinuria [31, 33, 34, 44, 45], increased oxidative stress [96, 97], mitochondrial dysfunction [96, 98], hyperhomocysteinemia [99–101], functional vitamin B6 deficiency [102–104], hyperoxalemia and hyperoxaluria [104, 105], and hypomagnesemia [64, 106]. Both porphyria and ASD exhibit enhanced pyridoxal 5'-phosphate-dependent tryptophan-kynurenine metabolism, probably mediated by substrate availability, at the expense of the tryptophan-serotonin-melatonin pathway [103, 107]. Increased oxidative stress in porphyria is evident by increased malondialdehyde [108] and may be partly produced by increased  $\delta$ -aminolevulinic acid levels [109]. The presence of mitochondrial dysfunction in porphyria is supported by reduced total cellular ATP levels [110], reduced oxygen consumption rate [96], and reduced expression of mitochondrial respiratory chain proteins [108, 110].

Alanine-glyoxylate aminotransferase (AGT) is a pyridoxal-5'-phosphate-dependent enzyme involved in the metabolism of glyoxylate [111]. Depletion of pyridoxal-5'-phosphate may cause hyperoxalemia and hyperoxaluria due to reduced activity of AGT. Defects in porphyrin metabolism can affect the transsulfuration pathway of sulfur amino acid metabolism leading to hyperhomocysteinemia [99]. This interrelationship could be partly explained by the knowledge of cofactors and regulation of cystathionine beta-synthase (CBS), catalyzing the first committed step. Haem, the synthetic product of porphyrin metabolism, is also a cofactor for the cystathionine beta-synthase enzyme [112]. Vitamin B6 is a cofactor for delta-aminolevulinic acid synthase 1 (ALAS1), the rate-limiting step of haem biosynthesis, and

CBS and cystathionine-gamma lyase (CGL) in the trans-sulfuration pathway. CBS has a lower affinity for pyridoxal 5'-phosphate compared to ALAS1 and other pyridoxal 5'-phosphate-dependent enzymes in the tryptophan-kynurenine pathway and therefore is at a disadvantage when pyridoxal 5'-phosphate is decreased [112]. The plausible metabolic consequences of abnormal porphyrin metabolism in ASD are illustrated in Fig. 1.

CBS activity can be also reduced in haem deficiency, interfering with pyridoxal 5'-phosphate binding and promoting CBS degradation [112]. Therefore, hyperhomocysteinemia in porphyria patients is most likely to occur from decreased hepatic CBS and CGL activity due to low haem availability, as well as possible relative/absolute vitamin B6 deficiency caused by ALAS1 induction [99]. Considering the evidence at hand, deciding whether atypical porphyrin excretion in ASD is a cause or consequence is premature. The link between sulfur amino acid metabolism and porphyrin metabolism is further corroborated by a study demonstrating that compared to unaffected controls, compound heterozygotes for *ALAD* 177CG and *RFC1* 80AG variants were nearly four times more likely to be autistic. *RFC1* encodes a reduced folate carrier involved in the folate transport, which is linked to the remethylation pathway of the methionine cycle [75]. Moreover, the same research group demonstrated that a significant lowering of plasma reduced glutathione and glutathione redox ratio in ASD children with

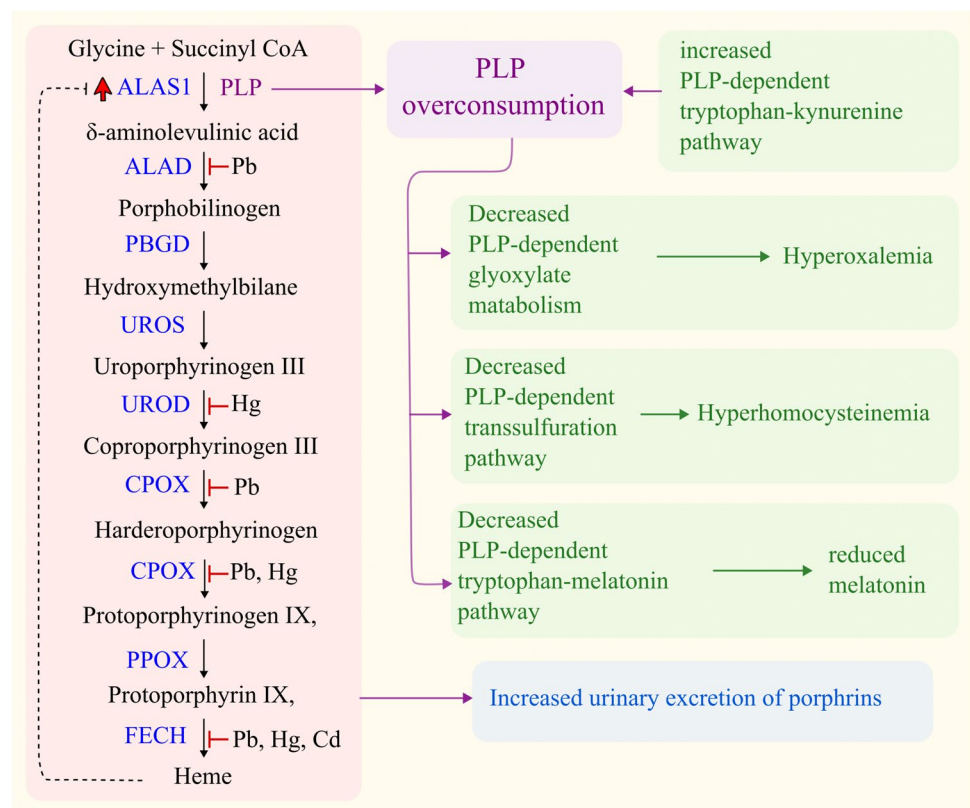
*ALAD2* allele compared to controls [75]. Despite adult acute porphyrias predominantly affecting females, pediatric-onset acute hepatic porphyria shows a male predominance [91], corresponding with the male predominance well-established in ASD [4].

## Significance of Abnormal Thiol and Selenol Metabolism

Even though the underlying pathogenic mechanism of abnormal porphyrin metabolism is yet to be uncovered, the present understanding of metabolic interrelationships provides intriguing clues to a possible connection between abnormal thiol and selenol metabolism. Altered sulfur metabolism has received much attention due to consistently reported abnormalities corroborated by meta-analyses. Subjects with ASD have lower levels of methionine, cysteine, vitamin B12, folate, and S-adenosylmethionine to S-adenosylhomocysteine ratio [101]. In addition, the total thiol concentrations, median reduced thiol ratios, and mean native thiol were significantly lower. In contrast, median oxidized thiol ratios, redox potential, and median disulfide concentrations were considerably higher in children with ASD than in healthy controls [113].

Interestingly thiol groups in free cysteine, cysteine residues of proteins, selenol groups of seleno-cysteine, and

**Fig. 1** Hypothesized mechanism for abnormal porphyrin metabolism and its metabolic consequences in ASD. Heavy metals may block several steps in haem biosynthesis resulting in porphyrinuria. Overconsumption of pyridoxal 5'-phosphate (PLP) by induced ALAS1 and PLP-dependent enzymes in the tryptophan-kynurenine pathway leads to reduced activity of other PLP-dependent enzymes, hence abnormal homocysteine, oxalate, and melatonin levels. (ALAS1; delta-aminolevulinic acid synthase 1, ALAD; delta-aminolevulinic acid dehydratase, PBGD; porphobilinogen deaminase, UROS; Uroporphyrinogen-III synthase, UROD; Uroporphyrinogen decarboxylase, CPOX; Oxygen-dependent coproporphyrinogen-III oxidase, PPOX; Protoporphyrinogen oxidase, FECH; Ferrochelatase)



seleno-cysteine residues of selenoenzymes and other selenoproteins are critical targets of Hg toxicity. The binding of Hg compounds to thiol and selenol groups depletes these substances and inhibits antioxidant enzymes, which are essential to mitigate oxidative damage [114]. Moreover, the effects of heavy metals on enzymes involved in thiol and selenol metabolism are brain region-specific especially affecting the hypothalamus and brainstem structures [115]. The sulfur-containing ligands exhibit protective and reactivation effects toward the enzyme inhibition [116].

Hg is a well-known toxic heavy metal that occurs in several forms, i.e., metallic Hg, Hg vapor, mercurous ions, mercuric ions, and organo-Hg compounds (e.g., methyl-Hg). Food contaminated with Hg, dental amalgams, Hg-containing cosmetics, and Hg-containing paints are some of the common sources of Hg exposure in humans [117]. The most toxic form, methyl-Hg, is readily absorbed via biological membranes such as gut and placenta due to its lipophilic nature [118]. It is easily complexed with cysteine to produce an abnormal amino acid analogous to methionine. Methyl-Hg competes with amino acids for transport via specialized amino acid transporters at the placenta and blood-brain barrier and accumulates in the brain [86]. These harmful actions of Hg are mitigated by glutathione, a naturally occurring chelator, that serves as the major detoxification mechanism of heavy metals (e.g., Hg and Pb) [75].

Interestingly pre-coproporphyrin and penta-carboxylporphyrin levels have shown a positive correlation with plasma-oxidized glutathione levels in subjects with ASD [42]. Depletion of thiols such as glutathione may predispose to increased susceptibility to heavy metal toxicity and resultant atypical urine porphyrin profile in subjects with ASD. The deficiency of thiols may reduce biliary and renal excretion of heavy metals, increasing their toxicity. Whole

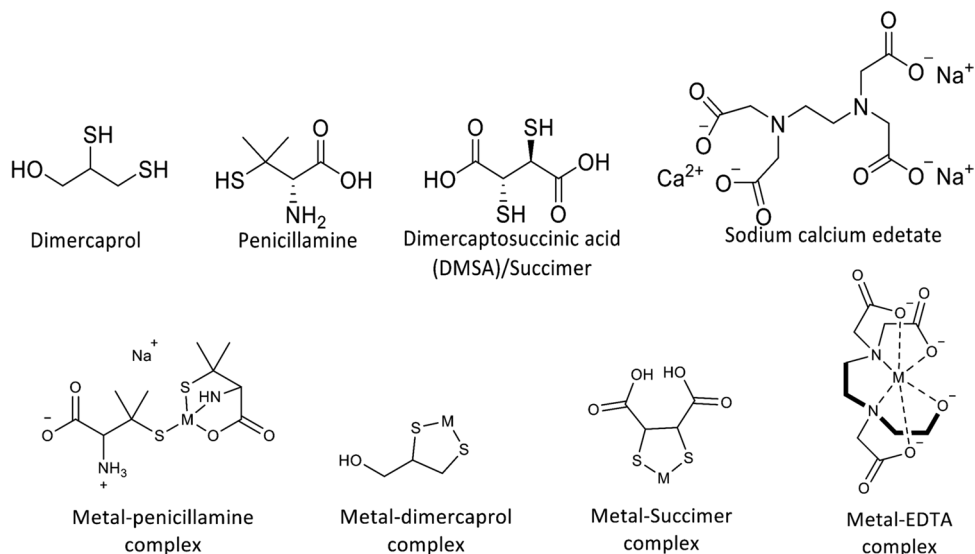
blood Hg levels were significantly higher in ASD patients compared to healthy subjects. The Hg level in hair was significantly lower in healthy subjects, whereas there was no difference in the urinary Hg level. This could be attributed to the evidence of an impairment in the detoxification and excretory mechanisms in ASD [119].

Cellular accumulation of oxalate (which is associated with abnormal haem metabolism, as discussed above) [96, 105] may also contribute to mitochondrial dysfunction and oxidative stress [120]. A significant proportion of cellular oxalate binding appears to occur in the mitochondria and the oxalate binding increases with thiol depletion [121]. Additionally, oxalate-induced mitochondrial dysfunction in circulating monocytes appears to alter plasma cytokine and chemokine levels [122]. In light of these findings, oxalate appears to connect many dots in the intricate pathogenic mechanism of ASD.

## A metabolic Approach to the Treatment of ASD Associated with Porphyrinuria

Several chelating agents are used in clinical practice to manage heavy metal poisoning; dimercaprol, penicillamine, sodium calcium edetate, ethylenediaminetetraacetic acid (EDTA), succimer, etc. These agents bind to heavy metals (Pb, Hg, As) and facilitate renal excretion. Sulfhydryl moiety is responsible for the chelation of the metal ion forming a metal-sulfur linkage (Fig. 2) [123–127]. However, based on a Cochrane systematic review, there is no evidence that multiple rounds of oral DMSA would have an impact on ASD symptoms. On the other hand, chelation therapy is associated with serious adverse effects such as hypocalcemia, renal impairment, and even death. Therefore, the risks of using

**Fig. 2** Chemical structures of metal chelating agents and their metal complexes. EDTA; ethylenediaminetetraacetic acid





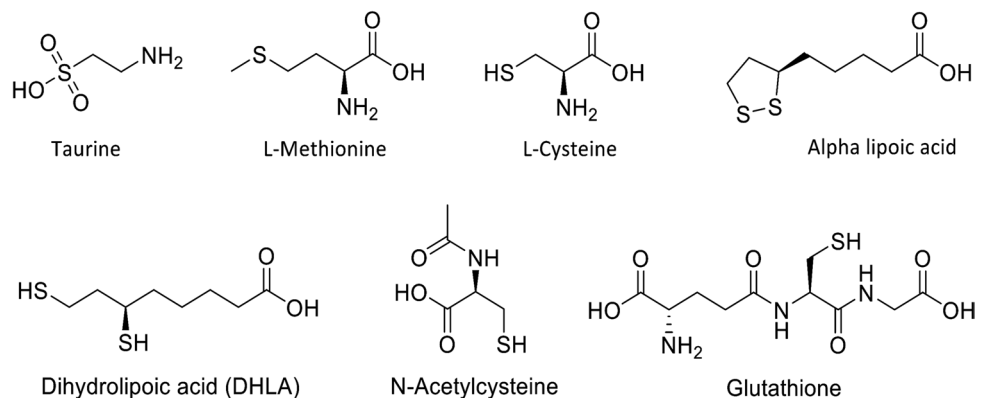
chelation for ASD treatment currently outweigh the proven benefits [128].

Several other supplements that can enhance natural chelation detoxification pathways, such as taurine, methionine, cysteine, alpha-lipoic acid and dihydrolipoic acid (DHLA), glutathione, and N-acetylcysteine (a precursor of glutathione), have received attention in addressing metal toxicities [116, 129]. Some of these agents, such as oral N-acetyl cysteine and oral alpha-lipoic acid are proposed as potential metabolic therapies that can be used in ASD associated with mitochondrial dysfunction [130]. Liposomal (absorbed better) and transdermal glutathione have shown benefits in improving plasma cysteine and reduced glutathione levels in subjects with ASD [131]. Evidencing metal chelation property, these compounds contain sulfhydryl moiety (Fig. 3), which can effectively chelate heavy metal ions of Hg and Pb. Notably, both reduced and oxidized forms of alpha-lipoic acid are known to chelate metal ions. At the same time, DHLA is a potent antioxidant in the body that can recycle other antioxidants like glutathione and ascorbic acid

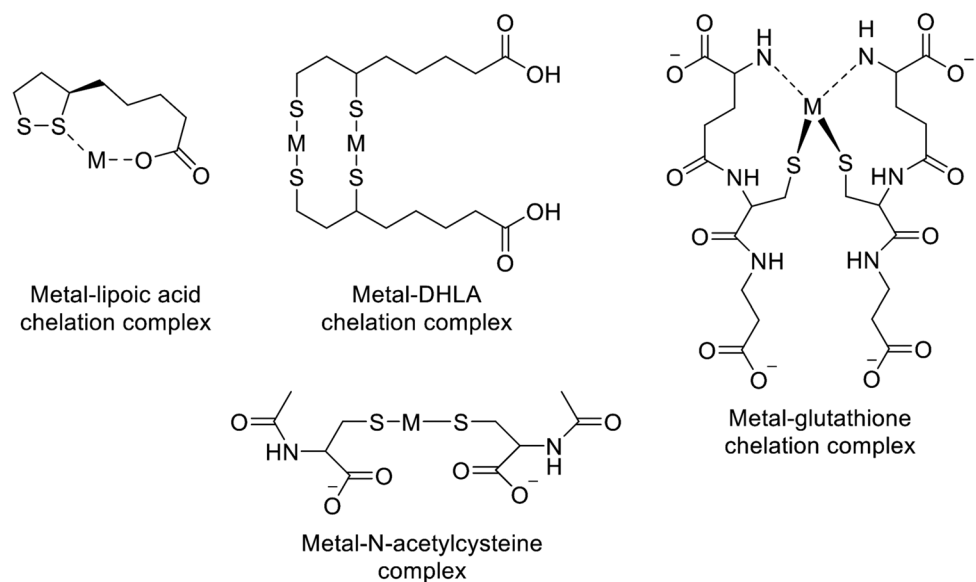
[132, 133]. Glutathione, N-acetylcysteine, and alpha-lipoic acid chelate heavy metals by forming a metal-sulfur linkage [134, 135] (Fig. 4). Additionally, alpha-lipoic acid can ameliorate mitochondrial dysfunction and increase ATP synthesis in cells with defective porphyrin metabolism [110]. The naturally occurring compounds with chelation properties are generally associated with minimal side effects and hence appear to be a good alternative to chelating agents associated with severe adverse effects such as DMSA. Moreover, calcium and iron supplementation attenuate Pb accumulation, while dietary deficiencies are reported to enhance Pb absorption. This forms the basis for using calcium and iron supplementation to manage Pb poisoning [136].

Nevertheless, the action of glutathione on brain Hg levels depends on the form of Hg. For instance, glutathione plays a crucial role in methylmercury transport into the brain. Watanabe et al. demonstrated that the transport of methylmercury into the brain is accelerated by glutathione, but retarded by the surplus glutathione. It was further suggested that methylmercury is transported into the brain after being converted

**Fig. 3** Naturally occurring compounds possessing metal chelation and antioxidant properties



**Fig. 4** Metal complexes formed by some natural chelators. Letter M in chemical structures indicates a heavy metal iron. DHLA, dihydrolipoic acid



to methylmercury-cysteine by gamma-glutamyl transpeptidase [137]. Conversely, elemental mercury uptake by the brain is increased by depletion of glutathione in rats [138]. DMSA, 2,3-dimercaptopropane-1-sulfonic acid (DMPS), glutathione, vitamin C, lipoic acid alone, or in combination were able to reduce kidney Hg levels but unable to reduce elementary Hg content of the brains of rats exposed to Hg [139, 140]. On the other hand, sulfhydryl compounds appear to have non-chelation functions such as free radical scavenging ability and protective effects against uroporphyrinogen-decarboxylase inhibition [141] that may be more relevant in the subset of ASD with abnormal porphyrinuria.

A study revealed that thiamin and thiamin monophosphate (TMP) concentrations are not significantly different from healthy subjects, while thiamin pyrophosphate (TPP) levels were decreased by 24% in children with ASD [142]. Moreover, dysautonomia associated with abnormal erythrocyte transketolase has been observed in two ASD individuals, indicating abnormal thiamin homeostasis. [143]. The use of thiamin in the treatment of ASD has mainly been due to its function as a cofactor in the citric acid cycle enzymes [130]. In one pilot study, two months of thiamine tetrahydrofurfuryl disulfide (TTFD) supplementation has shown clinical improvement in eight out of the ten children with ASD as measured by Autism Treatment Evaluation Checklist (ATEC), with an increase in urinary Cd and Pb observed in two children and one child, respectively [144]. Concomitant thiamine administration with chelators such as EDTA enhanced the efficacy by potentiating urinary Pb excretion, reducing tissue Pb including brain Pb, and restoring Pb-induced biochemical alterations, suggesting a promising role of thiamine as a complementary agent to heavy metal chelators [145]. Thiamin interacts with the pyrimidine ring, forming a readily excretable metal Pb-thiamine complex [146]. Facilitation of cellular penetration by EDTA and increasing effective chelation of intracellular bound Pb is another proposed mechanism [145]. Interestingly, thiamin is also a sulfur-containing molecule similar to most metal chelators. Importantly thiamin is a safe nutritional supplement with no adverse effects [130] but many beneficial metabolic effects in ASD.

Sulforaphane is a sulfur-containing compound found in cruciferous vegetables such as cauliflower, broccoli, and cabbage. It is a dietary supplement with minimal side effects. It has multiple biological effects pertinent to ASD, including antioxidant, anti-inflammatory, and neuroprotective effects. A systematic review of five clinical trials demonstrated a significant positive correlation between sulforaphane use and improvement in ASD behavior and cognitive function [147]. Metallothionein,

a primary antioxidant enzyme involved in the metabolism and detoxification of heavy metals, is induced by sulforaphane through modulation of the *MT* gene expression [148]. Corroborating these findings, the administration of sulforaphane has been found to increase the brain glutathione levels [149].

The presence of hyperhomocysteinemia, hyperoxalemia/hyperoxaluria, vitamin B6 deficiency, and magnesium (Mg) implies that a vitamin B6, Mg, and low oxalate diet might be effective. Vitamin B6 will enhance the activity of vitamin B6-dependent enzymes such as CBS and thereby correct hyperhomocysteinemia. As many B6-dependent enzymes use Mg also as a co-factor, vitamin B6-Mg co-therapy appears to be a good combination [102]. On the other hand, vitamin B6 also has non-cofactor functions as a metal chelator and potent antioxidant, pertinent in ASD [150].

Additionally, lower blood Zn levels [53, 64] and elevated blood Cu/Zn ratios [35] were also found in ASD individuals. Therefore, it has been hypothesized that Hg toxicity-associated metallothionein dysfunction may be one of the contributory factors of Zn deficiency in children with ASD [151]. Therefore, Zn deficiency might potentiate the toxic effects of heavy metals even at a low level of exposure [65]. Zinc supplementation may be used as a possible preventative and therapeutic approach to lessen the core/co-occurring symptoms associated with ASD [102].

Another promising therapeutic approach in porphyrinuria-associated ASD is melatonin which has received attention in ASD [152] as well as in porphyria [153] and may be mediated by scavenging reactive oxygen species and suppression of the key enzyme ALAS1 [153]. Additionally, melatonin has been shown to reverse Pb-induced neurotoxicity in animal models by eliciting its properties via antioxidants and other mechanisms [154].

Multiple levels of evidence suggest a possible role of heavy metals, impaired detoxification pathways in the pathogenesis of autism, impaired porphyrin metabolites as potential biomarkers, and chelating agents, thiols, thiamin, and sulforaphane as potential therapeutic agents. The abnormal porphyrin metabolites appear to be candidate biomarkers useful in precision medicine in ASD. However, despite all that attention given to the heterogeneity of ASD, many studies compared the effectiveness of the heavy metal-targeted interventions between chelated-ASD subjects vs. non-chelated-ASD subjects. Future research should focus on comparing the efficacy of less invasive nutritional approaches in reducing co-symptoms and co-occurring symptoms in the subgroup of patients with increased porphyrin excretion.

## Concluding Remarks

Porphyrin metabolites are potential biomarkers for ASD, having relatively low sensitivity but very high specificity. Their levels were positively correlated with the severity of autism. The low sensitivity is attributed to the high heterogeneity of this group of disorders with a broad spectrum of underlying pathogenic mechanisms. Heavy metal poisoning was proposed as one of the causative factors, as heavy metals are known inhibitors of several metabolic pathways. An abnormal urinary porphyrin profile was reported to correlate with ASD, and exposure to heavy metals may play a role. Several chelating agents have been used in clinical practice to manage heavy metal poisoning, but the risks of their use outweigh the proven benefits. Many supplements with therapeutic benefits in ASD, such as N-acetyl cysteine, alpha-lipoic acid, thiamin, sulforaphane, melatonin, and zinc appear to have potential benefits in the subgroup of ASD with abnormal porphyrin metabolism. However, more research into the role of abnormal porphyrin metabolism in ASD is needed to translate scientific evidence into clinical practice.

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**Data Availability** Not applicable

## Declarations

**Ethics Approval** Not applicable

**Consent to Participate** Not applicable

**Consent for Publication** Not applicable

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