*Original Research*

# Decreased glutathione and elevated hair mercury levels are associated with nutritional deficiency-based autism in Oman

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## Abstract

Genetic, nutrition, and environmental factors have each been implicated as sources of risk for autism. Oxidative stress, including low plasma levels of the antioxidant glutathione, has been reported by numerous autism studies, which can disrupt methylation-dependent epigenetic regulation of gene expression with neurodevelopmental consequences. We investigated the status of redox and methylation metabolites, as well as the level of protein homocysteinylation and hair mercury levels, in autistic and neurotypical control Omani children, who were previously shown to exhibit significant nutritional deficiencies in serum folate and vitamin $B_{12}$. The serum level of glutathione in autistic subjects was significantly below control levels, while levels of homocysteine and S-adenosylhomocysteine were elevated, indicative of oxidative stress and decreased methionine synthase activity. Autistic males had lower glutathione and higher homocysteine levels than females, while homocysteinylation of serum proteins was increased in autistic males but not females. Mercury levels were markedly elevated in the hair of autistic subjects vs. control subjects, consistent with the importance of glutathione for its elimination. Thus, autism in Oman is associated with decreased antioxidant resources and decreased methylation capacity, in conjunction with elevated hair levels of mercury.

**Keywords:** Cobalamin, epigenetic, folic acid, homocysteine thiolactone, methionine synthase, neurodevelopment

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## Introduction

The prevalence of autistic spectrum disorders (ASD) has increased dramatically in countries around the world over the past several decades, reaching a prevalence in the USA of 1 in 88, based upon 2008 data.[1] While this group of neurodevelopmental disorders is primarily identified by neurological symptoms,[2] the reported co-occurrence of immune dysfunction[3–10] and gastrointestinal symptoms[8,10–14] with ASD indicates that it is not strictly limited to brain dysfunction. The increase in ASD has stimulated intense research into potential etiologic factors and candidate genes. Current research indicates that autism involves an interaction between genetic factors[15–18] and environmental factors,[19–21] manifested as epigenetic dysregulation.[22–24]

Epigenetic regulation of gene expression is increasingly recognized as a fundamental aspect of development, and abnormalities affecting DNA methylation and its epigenetic influence are linked to neurodevelopmental disorders, including Rett and Angelman syndromes, Fragile-X disorder and autism.[25–27] Like other methylation reactions, DNA methylation depends upon the ratio of the methyl donor S-adenosylmethionine (SAM) to the methylation inhibitor S-adenosylhomocysteine (SAH), and the SAM/SAH ratio is in turn dependent upon activity of the folate and vitamin $B_{12}$-dependent enzyme methionine synthase (MS). MS activity and methylation capacity are highly sensitive to cellular redox status (i.e. the probability of reduction vs. oxidation) and are inhibited under oxidative stress

conditions.[28] More than 10 studies have reported that plasma levels of the antioxidant glutathione (GSH) are lower in autistic children, accompanied by a decrease in the SAM to SAH ratio.[15,29–37] Low methylation capacity and oxidative stress are associated with individuals with low levels of folate and vitamin $B_{12}$.[38–40] Thus, impaired methylation capacity, including DNA methylation, can result from either oxidative stress or a nutritional deficiency in folate or vitamin $B_{12}$.

A recent analysis of ASD children in Oman revealed that malnutrition was common and serum levels of folate and vitamin $B_{12}$ were significantly lower, in association with low dietary intake.[41] Since biomarkers of oxidative stress were elevated in this cohort,[42] we analyzed serum levels of a panel of antioxidant and methylation pathway metabolites, which prior studies showed to be abnormal in ASD subjects.[15,29] Since autism is more prevalent among males vs. females, including in Oman,[43] we also included a gender-specific analysis for control and ASD cohorts. Decreased MS activity is reflected as elevated homocysteine (HCY) levels, which can lead to increased N-homocysteinylation of proteins,[44] so we therefore evaluated the N-homocysteinylation status of serum albumin. Hair samples in this ASD cohort were previously shown to have elevated levels of several heavy metals.[45] Mercury is well-recognized for its ability to interfere with antioxidant regulation, including both GSH and selenium-based systems,[46,47] although its proposed involvement in autism remains controversial.[22,48,49] Therefore, we also measured mercury levels in hair samples of ASD vs. control subjects, comparing them to thiol metabolite levels.

**Table 1** Socio-demographic characteristics of ASD and control groups

| Characteristics | Cases ($N=27$) N (%) | Controls ($N=27$) N (%) | P value |
|---|---|---|---|
| Gender | | | 0.51 |
| Male | 22 (81.5) | 20 (74.1) | |
| Female | 5 (18.5) | 7 (25.9) | |
| Mean age (year) | 5.3 (1.5) | 5.5 (1.4) | 0.84 |
| Mean birth weight (kg) | | | |
| 2.50–3.99 | 23 (85.2) | 21 (77.8) | 0.12 |
| <2.50 | 3 (11.1) | 5 (18.5) | |
| >3.99 | 1 (3.7) | 1 (3.7) | |
| Area of residence | | | 0.31 |
| Urban | 19 (70.4) | 17 (63.0) | |
| Rural | 8 (29.6) | 12 (44.4) | |
| Monthly family income (OR) | | | 0.39 |
| Less than 500 | 11 (40.7) | 9 (33.3) | |
| 500 to 1000 | 10 (37.0) | 12 (44.4) | |
| Greater than 1000 | 6 (22.2) | 6 (22.2) | |
| Educational level of mother | | | 0.24 |
| Illiterate | 3 (11.1) | 5 (18.5) | |
| Basic education | 15 (55.6) | 14 (51.9) | |
| Finished high school | 9 (33.3) | 8 (29.6) | |
| Occupation of mother | | | 0.31 |
| Working | 10 (37.0) | 8 (29.6) | |
| Housewife/ retired | 17 (63.0) | 19 (70.4) | |

## Materials and methods

### Participants

Study participants included 54 Omani children (27 ASD cases and 27 control subjects). A demographic comparison of the two groups showed a similar age and gender distribution, with a preponderance of male subjects (Table 1). The study was approved by the Medical Research Ethics Committee at Sultan Qaboos University (SQU) and by the Northeastern University Institutional Review Board (Protocol #01-02-06). All caregivers of the participants provided their written informed consent prior to inclusion in the study.

For ASD cases, ascertainment of ASD diagnosis was made according to the Childhood Autism Rating Scale (CARS), which was developed using gold-standard criteria based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR).[2] Accordingly, all participants fulfilled the eligibility for diagnosis of ASDs, exhibiting symptoms within the triad of typical autistic traits: communication impairment, social deficits, and ritualistic interests. Confirmed ASD cases from the SQU clinic were referred to study coordinators for potential participation in the study. All CARS scores and clinical evaluations were conducted and reviewed by behavioral medicine clinicians with long-standing experience in caring for children with autism. The reviewers developed and employed a coding guide based on the DSM-IV-TR criteria to determine if the child's condition was consistent with the standard international ASD diagnostic criteria. Inter-rater reliability was established among ASD clinician reviewers to standards of 90% agreement on overall case status. For ongoing inter-rater reliability checks, a random sample of records (10%) was scored independently by a reviewer who is experienced in ASD and did not participate in the diagnostic reviews. Percentage agreement between the raters on final case definition was found to be 96%.

Control subjects were randomly selected from eligible outpatients at the Department of Child Health at SQU. Eligible subjects included children aged 3–5 who were not known to have any overt neurodevelopmental or behavioral disturbances. Eligible diagnoses included trauma, routine physical examination, dental problems, and dermatological problems. Nutritional and serum status of vitamin B12 and folate for subjects in this study were previously reported.[42]

### Serum thiol and methionine cycle metabolites

Blood samples were collected and serum was stored at −80°C prior to assay. Serum thiols were measured using high performance liquid chromatography (HPLC) and electrochemical detection. Serum samples were thawed on ice and 50 μL of a 0.4 N perchloric acid solution was added to 200 μL of serum to precipitate proteins. Samples were blown with nitrogen to displace atmospheric air, and then

spun at 13,000 RPM on a tabletop microcentrifuge for 60 min at 4°C. Then 100 μL of sample was added to a conical micro-autosampler vial, blown with nitrogen, capped, and kept at 4°C in the autosampler (ESA model 542) cooling tray. Later, 10 μL of sample was injected into an ESA CoulArray HPLC system with a BDD analytical cell (model 5040) electrochemical detector at an operating potential of 1500 mV, equipped with an Agilent Eclipse XDB-C8 (3 × 150 mm, 3.5 μm) reverse-phase C8 column. A dual mobile phase gradient elution was used, consisting of a mobile phase containing sodium phosphate 25 mM and 1-octanesulfonic acid 2.1 mM, adjusted to pH 2.65 with phosphoric acid, with the second mobile phase containing 50% acetonitrile. The system was run at a flow rate of 0.6 mL/min at ambient temperature with the following gradients: 0–9 min 0% B, 9–38 min, gradient to 30% B. Post-run, the system was cleaned 38–42 min with 100% B and the cell was cleaned 39–42 min at an operating potential of 1900 mV. The system was allowed to equilibrate at 0% B from 42 to 60 min. Peak area analysis was provided by CoulArray 3.06 software (ESA, Chelmsford, Massachusetts) based on the standard curves generated for each compound.

### Protein N-homocysteinylation

Serum proteins (1.3 mg/mL) were incubated with 250 μM biotin-aldehyde in 100 mM citric acid, 2.7 mM tris(2-carboxyethyl)phosphine (TCEP), pH 3 in the dark, at 25°C for 12 h. Then, 35 μL of each sample was mixed with 2X Laemmli loading buffer (35 μL) in boiling water for 5 min. Aliquots (30 μL) were loaded onto two precast Tris-HCl gels (4–15%, Bio-Rad) for SDS-PAGE separation. The first gel was used for Coomassie blue staining and the second for western-blot detection as previously described.[50] Proteins from the gel were transferred onto an Immun-Blot PVDF Membrane (0.2 μm, Bio-Rad) for protein blotting with transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol at pH 8.3). After protein transfer, the membrane was blocked in 2% BSA in TBST (25 mM Tris, 137 mM NaCl, 3 mM KCl, 0.1% Tween-20 at pH 7.4) for 1 h. After blocking, the membrane was washed with TBST for 3 × 10 min and incubated with 0.5 μg/mL streptavidin-HRP in 20 mL TBST for 1 h. Then, the membrane was washed again by TBST for 5 × 6 min and incubated in PBS (68 mM NaCl, 1 mM KCl, 5 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$ at pH 7.4) for 10 min. After incubation, the buffer was discarded, and the chemiluminescence signal was developed by the addition of 1 mL SuperSignal West Pico chemiluminescent substrate for 1 min. Chemiluminescence was detected by FluorChem Imager SP (Alpha Innotech Corp., San Leandro, CA, USA), and the image was analyzed by ImageQuant TL 7.0 (GE Healthcare). The protein N-homocysteinylation level of selected proteins was calculated from the chemiluminescence intensity of each band divided by the Coomassie staining intensity of total proteins in the same lane, and final intensity was normalized according to the intensity of total protein marker bands.

### Hair mercury analysis

Using stainless-steel scissors, the hair specimens were cut into approximately 0.3-cm pieces and mixed to allow a representative subsampling of the hair specimen. After cutting and weighing the combined specimens (range 30–80 mg ± 0.1 mg), each sample was washed four times with a 1:200 v/v dilution of Triton X-100, then rinsed with acetone and allowed to drain. Samples were then rinsed three times with ultrapure deionized water and two times with acetone. The dried samples were weighed prior to nitric acid/microwave digestion. After digestion, the samples were cooled and a 500-μL aliquot of an internal standard was added and mixed with 50 mL ultrapure, deionized water. The samples were then analyzed for element content using inductively coupled mass spectrometry (ICPMS). Individual results are the means of three replicates, expressed as micrograms of mercury per gram of hair, and differences between replicate values were less than 8% of the mean value. Accuracy of the ICPMS methodology was verified by the appropriate use of reagent blanks, independent calibration verification standard check solutions, and a certified hair reference control (Trace Elements In Human Hair, CRM-397) obtained from the Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium). The rationale and utility of this procedure for identifying ASD-related differences in hair mercury levels have been described elsewhere.[51]

### Data analysis

Combined ASD vs. control group data, as well as gender-grouped data were analyzed. Grouped data were initially evaluated by ANOVA followed by post hoc analysis of individual metabolites using Student's t-test to evaluate significance, with a cut-off $P < 0.05$. All statistical analyses were performed using GraphPad Prism software version 5 (GraphPad Software Inc., La Jolla, CA, USA). Results are presented as average values ± S.E.M.

## Results

### Subject characteristics

Sociodemographic and nutritional characteristics of the ASD and control groups were previously described.[42,45] Nutritional intake and serum levels of folate and vitamin $B_{12}$ were found to be significantly lower in the ASD cohort.[42]

### Serum thiol and methionine cycle metabolites

Folate and vitamin $B_{12}$ are essential cofactors for MS, whose activity affects both methylation and transsulfuration pathways, providing an importance influence over epigenetic and redox status. To evaluate changes in these pathways in autism, we measured serum levels of thiols and methionine cycle intermediates via HPLC with electrochemical detection. As illustrated in Figure 1(a), significant differences were observed for several metabolites between combined control and autistic subjects. The level of GSH was significantly decreased in the autism group, being 71% lower than the control group ($P < 0.05$). Cystine, HCY, and SAH levels

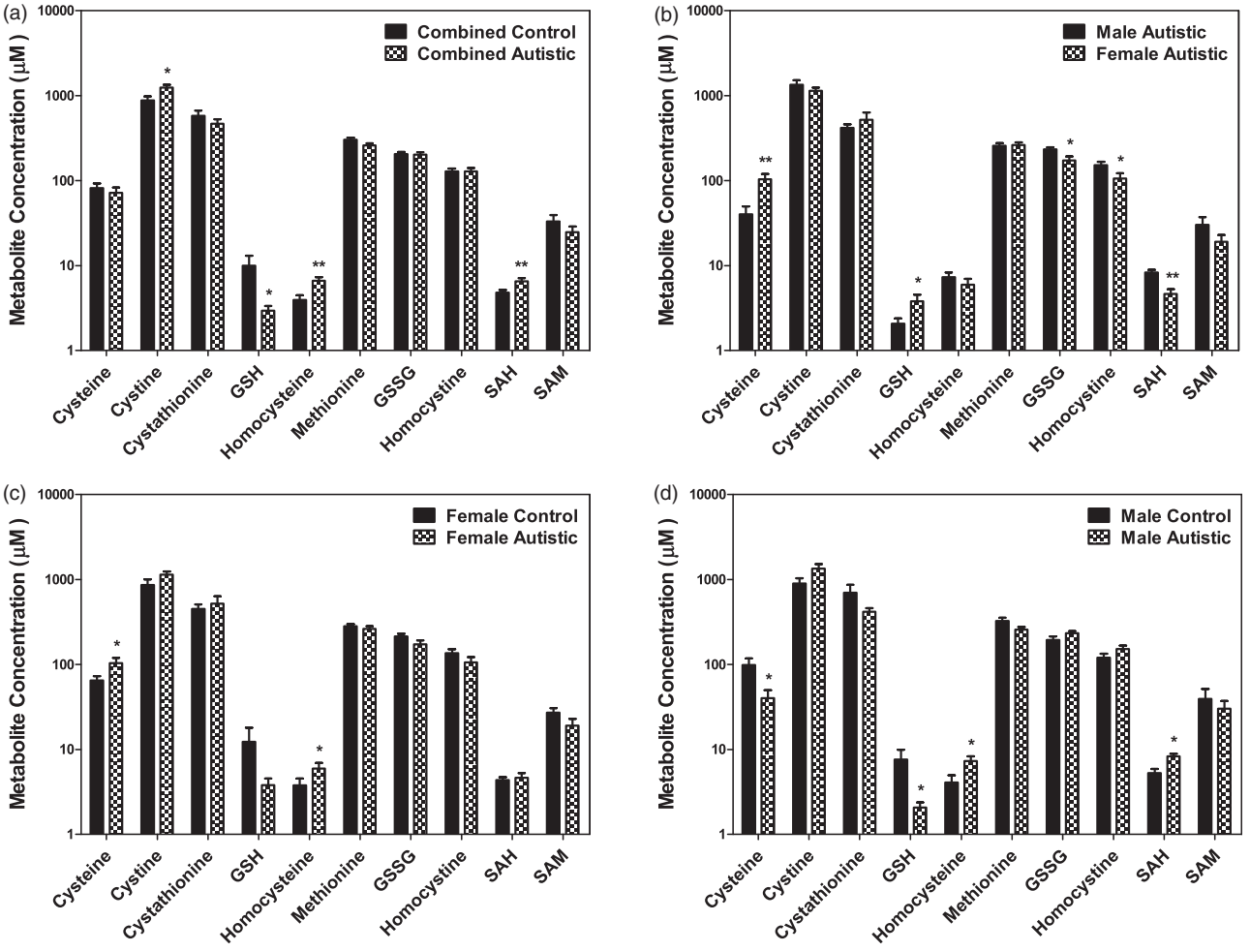


**Figure 1** Serum concentrations of redox and methylation metabolites in autism. Serum samples were analyzed by HPLC with electrochemical detection. (a) Metabolite levels in combined autistic vs. combined control subjects ($N = 30$ in each group). (b) Metabolite levels in male vs. female autistic subjects ($N = 15$ in each group). (c) Metabolite levels in autistic vs. control female subjects ($N = 15$ in each group). (d) Metabolite levels in autistic vs. control male subjects ($N = 15$ in each group). * and ** indicate significant differences between groups of $P < 0.05$ and $P < 0.01$, respectively

were significantly increased in the autism group, being 41% ($P < 0.05$), 68% ($P < 0.01$) and 36% ($P < 0.01$) higher than control group values, respectively. While the SAM to SAH ratio, indicative of methylation capacity, was lower in autistic subjects ($4.4 \pm 0.64$) than control subjects ($6.28 \pm 1.00$), the difference was not significant ($P = 0.13$).

Further analysis revealed significant gender-based differences in thiol and methionine cycle intermediates for autistic subjects. Although there was no significant difference in metabolite levels between control males and females (data not shown), autistic males had significantly lower levels of cysteine ($P < 0.01$) and GSH ($P < 0.05$) vs. their autistic female counterparts, as well as higher levels of GSSG ($P < 0.05$), homocystine ($P < 0.05$), and SAH ($P < 0.001$) (Figure 1b). While cysteine levels were significantly higher for females autistic subjects vs. female controls ($P < 0.05$) (Figure 1c), they were lower in male subjects vs. male controls ($P < 0.05$) (Figure 1d). SAH was significantly elevated in autistic males vs. controls ($P < 0.01$), but not in females. GSH was significantly lower for autistic males ($P < 0.05$), but not for females ($P = 0.15$), although the non-significant decrease for females was large (69%). Thus, male autistic subjects exhibit a more severely disturbed metabolic profile than females.

## Protein N-homocysteinylation

During new protein synthesis, HCY competes with methionine and lysine for binding to methionyl- or lysyl-tRNA synthetases and while it is bound to these tRNA synthetases, HCY is converted to HCY thiolactone (HCY-TL).[44,52] HCY-TL readily reacts with lysine residues in proteins, with the potential for altering their function and antigenicity, as well as introducing a new thiol moiety. Decreased methionine synthase activity increases HCY-TL formation, as well as the level of protein N-homocysteinylation. HCY-TL is inactivated by paraoxonase 1, a target of organophosphate pesticides, whose activity is decreased in autistic subjects.[53,54]

We developed an assay for quantification of protein N-homocysteinyation, based upon chemical modification followed by biotinylation.[50] Using this assay, we evaluated the N-homocysteinylation status of five selected proteins in serum samples from autistic and control subjects, as

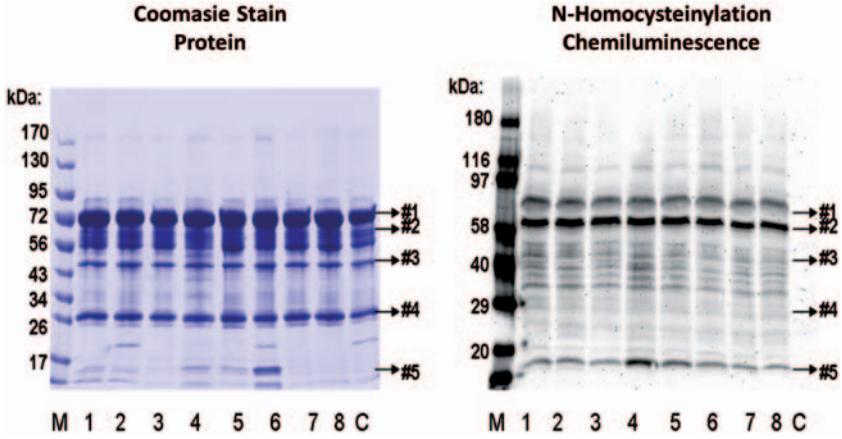


**Figure 2** Homocysteinylation of serum proteins. Serum proteins were separated by electrophoresis in duplicate gels. One gel was stained with Coomasie blue to evaluate protein density (left panel) and the second gel was utilized for evaluation of N-homocysteinylation intensity as chemiluminescence (right panel). Five specific protein bands were quantified and the level of N-homocysteinylation was compared for male and female autistic and control subjects, as provided in Table 1. In this representative example, sample lanes 1–4 are from autistic females and lanes 5–8 are from control males. M = molecular weight standards. (A color version of this figure is available in the online journal.)

**Table 2** Chemiluminescence intensity of serum protein N-homocysteinylation.

| Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|
| | Autism | Control | *P* value | | Autism | Control | *P* value |
| Band 1 | 1.08 ± 0.69 | 1.00 ± 0.30 | 0.7 | Band 1 | 0.87 ± 0.52 | 1.00 ± 0.77 | 0.6 |
| Band 2 | 1.35 ± 0.69 | 1.00 ± 0.24 | 0.08 | Band 2 | 0.78 ± 0.30 | 1.00 ± 0.66 | 0.3 |
| Band 3 | 1.40 ± 0.76 | 1.01 ± 0.37 | 0.1 | Band 3 | 0.73 ± 0.29 | 0.99 ± 0.71 | 0.2 |
| Band 4 | 1.26 ± 0.49 | 0.99 ± 0.36 | 0.1 | Band 4 | 1.05 ± 0.79 | 1.00 ± 0.34 | 0.9 |
| Band 5 | 1.74 ± 1.10 | 1.00 ± 0.48 | 0.03 | Band 5 | 0.90 ± 0.42 | 1.00 ± 1.00 | 0.7 |
| Overall | 1.40 ± 0.60 | 1.00 ± 0.28 | 0.03 | Overall | 0.87 ± 0.40 | 1.00 ± 0.66 | 0.6 |

illustrated in Table 2 and Figure 2. A comparison between groups indicated that the level of N-homocysteinylation was significantly higher for protein #5 and for the combined proteins in autistic males vs. control males ($P < 0.03$), but not for autistic females vs. control females (Table 1). There was no significant difference between combined male and female autistic subjects vs. combined control subjects.

### Hair levels of mercury

A previous study described elevations of several toxic metals in hair samples from our ASD cohort.[45] Consistent with this finding, we found that hair levels of mercury were markedly elevated in Omani autistic subjects ($6.93 \pm 0.36\,\mu g/g$) vs. control subjects ($0.611 \pm 0.033\,\mu g/g$), by more than 10-fold ($P < 0.0001$) (Figure 3a). The increase in mercury is greater than the increase observed for other toxic metals. There was no significant difference in mercury levels between male and female subjects within either group (Figure 3b). A comparison of hair mercury levels with serum thiol levels revealed a significant inverse correlation with serum levels of cysteine, cystine and GSSH in control subjects (Figure 3(c), (e) and (g)), but not in autistic subjects (Figure 3(d), (f) and (h)). No other significant correlations were found.

## Discussion

Autism is a disorder of neurodevelopment, but inflammation, autoimmune,[3–10] and gastrointestinal symptoms[8,10,11–14] are reported to also be present in autistic individuals, indicative of a systemic condition with prominent neurological manifestations. Prior studies have provided evidence of oxidative stress,[15,29–38] including significant deficits in plasma concentrations of GSH, the principal intracellular antioxidant. In confirmation of these studies, we also found low serum GSH levels in Omani ASD subjects, accompanied by elevated levels of methionine cycle intermediates HCY and SAH, and increased protein homocysteinylation in male ASD subjects. Furthermore, the content of mercury (Figure 3) and other heavy metals[45] in hair samples was markedly higher in ASD subjects. Together these observations serve to illustrate the pleiotropic roles of thiol metabolites, involving redox regulation, methylation reactions, and xenobiotic detoxification, each of which may potentially contribute to autism.

Adequate levels of GSH are essential to offset the effects of reactive oxygen species (ROS) released during aerobic metabolism, contributing to the maintenance of optimal metabolic activity.[55] Reciprocally, when GSH levels are inadequate, or the proportion of GSH to GSSG is low, aerobic metabolism is restricted, which decreases the risk of oxidative damage. The occurrence of mitochondrial

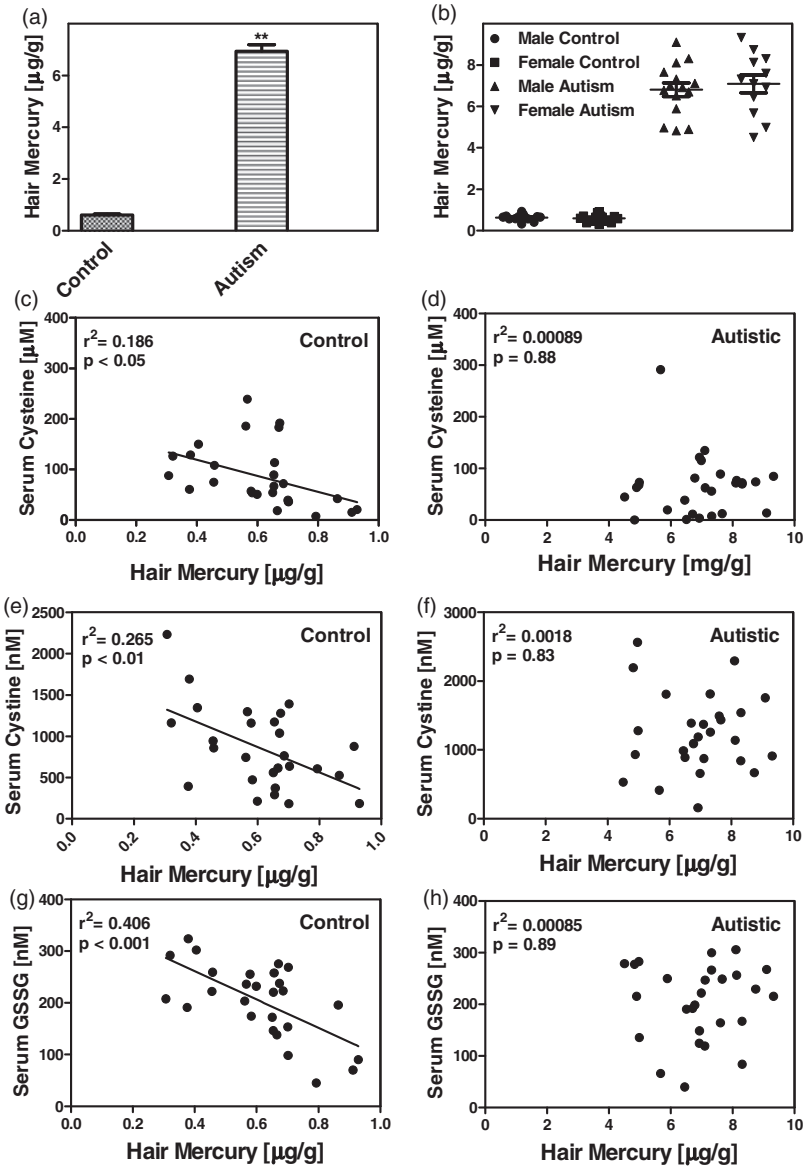


**Figure 3** Hair mercury levels in autism. Scalp hair samples were analyzed by mass spectrometry, as previously described (48). (a) Mercury levels in autistic vs. control subjects ($N = 30$ in each group). *** Indicates significant difference between groups ($P < 0.0001$). (b) Gender-based comparison of individual mercury levels ($N = 15$ in each group). (c–h) Correlation between hair mercury levels and serum cysteine (c, d), cystine (e, f), and GSSG (g, h) levels for control (c, e, f) and autistic (d, f, h) subjects. Statistically significant correlations were only observed for control subjects.

dysfunction in ASD[10] is consistent with restricted aerobic metabolism secondary to diminished antioxidant capacity. While this restriction is a useful adaptation to minimize the effects of oxidative stress, it can have adverse effects on development, a period of high energy demand, especially in the brain, whose rate of oxygen consumption is 10-fold higher than other tissues.[56] Several recent studies have reported decreased GSH in postmortem brain of ASD subjects,[57,58] along with decreased expression of enzymes required for its synthesis.[59] Low GSH levels found in Omani autistic subjects and other ASD populations around the world suggest that low antioxidant levels and oxidative stress may be core features of autism.

Methylation activity is highly sensitive to oxidative stress, reflecting inactivation of the vitamin B12 cofactor in MS, which serves as a sensor of intracellular redox status.[60] Decreased MS activity leads to an increase in both HCY and SAH, and SAH is a potent inhibitor of methylation reactions. Thus, oxidative stress can lead to a decrease in all methylation reactions, including methylation of DNA and histones, which combine to provide epigenetic regulation of gene expression.[61] Low levels of GSH have been previously shown to cause decreased DNA methylation.[28] It is increasingly clear that epigenetic regulation is the fundamental driving force for neurodevelopment.[62] Recent studies have revealed dynamic brain-specific patterns of DNA methylation[63] and hydroxymethylation[64,65] during postnatal development, and oxidative stress can disrupt these changes. Indeed, distinctive patterns of DNA methylation have been documented in blood cells postmortem brain of ASD subjects,[66,67] and we previously reported lower levels of MS mRNA in frontal cortex of ASD

subjects.[68] Thus, oxidative stress-induced changes in DNA methylation may contribute to impaired neurodevelopment in autism.

Accumulation of HCY upon MS inhibition can increase formation of HCY-TL, leading to increased formation of homocysteinylated proteins at exposed lysine residues.[69] Although the functional significance of homocysteinylation is poorly understood, it has been suggested that it might contribute to cardiovascular pathologies such as atherosclerosis, for example, secondary to homocysteinylation of LDL.[70,71] Homocysteinylation may compete with other N-terminal lysine modifications, such as methylation or ubiquitination, possibly affecting the rate of protein degradation. We observed increased homocysteinylation of serum proteins from ASD males but not females, suggesting that the intracellular ratio of HCY to methionine may be more elevated in autistic males vs. female autistic subjects, although serum (extracellular) levels of HCY were similarly elevated for both males and females cohorts (Figure 1(c) and (d)). Homocysteinylation of intracellular proteins might be a more sensitive indicator of HCY status.

The risk of autism is generally acknowledged to reflect both genetic and environmental factors.[19,22–24] Exposure to mercury and other heavy metals from multiple sources (e.g. fish consumption, air pollution, lead paint or the vaccine preservative thimerosal) can promote oxidative stress by interfering with GSH and selenoprotein-based antioxidant systems.[72,73] Selenoproteins have an almost irreversible affinity for mercury[74] and they play a more prominent antioxidant role in brain, as compared to other tissues,[75] making mercury is an extremely potent neurodevelopmental toxin.[76] Low nanomolar concentrations of methylmercury caused global DNA hypomethylation in neural stem cells, and this effect was carried forward into daughter cells which had not been directly exposed.[77] A study of mercury-exposed dental professionals did not find a correlation between hair mercury levels and global DNA methylation in buccal mucosa cells, but did find a significant negative correlation with methylation of the selenoprotein P1 locus in male subjects.[78] Uptake of selenoprotein P is a primary source of selenium for the brain.[79]

There have been conflicting reports about hair mercury levels in autistic children,[80,81] but several previous studies in the Middle East have found increased levels. A study of Kuwaiti children found 15-fold higher levels of mercury in autistic children,[51] while several studies of Saudi Arabian children found significant increases.[82,83] A recent comprehensive study of 1967 autistic subjects in Japan[84] found that a deficiency of essential minerals (zinc, magnesium, and calcium) accompanied by an excess of toxic metals (lead, aluminum, and cadmium) is common in autism, similar to our findings in Omani autistic subjects.[45] In the study of Yasuda et al.,[84] elevated hair mercury, defined as >2 standard deviations above a control reference value, was detected in only 2.8% of subjects. However, the reference value for their population was 3.87 μg/g, which is approximately 4-fold higher than our control subjects (0.61 μg/g). Our finding of a 10-fold higher mercury level in Omani autistic children may reflect a compromised ability to detoxify and excrete food-derived mercury. Since the per capita consumption of fish in Oman is 27.7 kg, compared to a global average of 16.7 kg, fish consumption may represent an importance of mercury. More detailed studies should be undertaken to establish the origin of mercury exposure.

GSH binds mercury and contributes to its detoxification, albeit with lower affinity, and lower GSH in autistic children may increase their vulnerability to mercury exposure, consistent with our finding that hair mercury levels correlated with cysteine, cystine, and GSSG in normal subjects, but not in autistic subjects. Thus, thiols represent a quantitatively important first line of defense against mercury, which limits the vulnerability of higher affinity selenoproteins, while thiol depletion may increase the opportunity for mercury to exert its neurotoxic effects.

Our findings are subject to several limitations. Importantly, we did not have access to data related to differences in mercury exposure between control and autistic subjects, so we cannot identify the cause of elevated hair mercury levels (i.e. increased exposure vs. decreased excretion). Since levels of other metals were also elevated in our ASD cohort,[45] increased exposure may be suspected, but the individual contribution of any single metal to autism cannot be readily evaluated. Lower levels of vitamin B12 and folic acid in this ASD cohort further confound analysis of the discrete role of mercury, and it is likely that both nutritional and environmental factors combine to increase the risk of autism.

In summary, GSH levels are significantly lower in the serum of autistic children in Oman, especially in males, and this decrease is accompanied by higher levels of mercury and other heavy metals in hair samples. In conjunction with previous results, these findings indicate that elevated heavy metal levels are a prominent feature of ASD in Oman.

**Author Contributions:** All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; NWH, MIW, MMA, OA, AO, and TZ conducted the experiments, RCD, AA, ZSZ, and YMA wrote the manuscript.

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