# Chinese Children with Autism: A Multiple Chemical Elements Profile in Erythrocytes

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Several lines of evidence suggested that abnormal levels of certain chemical elements may contribute to the development of autism spectrum disorders (ASD). The present work aimed to investigate the multiple chemical elements profile in the erythrocytes of autistic versus typically developing children (TDC) of China. Analyses were carried out to explore the possible association between levels of elements and the risk as well as the severity of ASD. Erythrocyte levels of 11 elements (32%) among 34 detected elements in autistic group were significantly different from those in the TDC group. To our knowledge, this is the first study which compared the levels of rare earth elements in erythrocytes between children with or without ASD. Five elements including Pb, Na, Ca, Sb, and La are associated with the Childhood Autism Rating Scale (CARS) total score. Also, a series of tendencies were found in this research which was believed to affect auditory response, taste, smell, and touch, as well as fear or nervousness. It can be concluded that Chinese autistic children suffer from multi-chemical element imbalances which involves a complex combination of genetic and environmental factors. The results showed a significant correlation between abnormal levels of several chemical elements and the severity of the autistic syndrome.

**Lay Summary**: It is suggested that abnormal levels of some chemical elements may contribute to the development of autism spectrum disorders (ASD). In this work, the impact of element imbalances on the risk and severity of ASD was investigated, focusing on the analysis of abnormal levels of the multi-chemical elements profile in erythrocytes compared with typically developing children. Furthermore, the results showed a significant correlation between abnormal levels of several chemical elements and the severity of the autistic syndrome. ***Autism Res*** *2018, 11: 834–845.* © 2018 International Society for Autism Research, Wiley Periodicals, Inc.

**Keywords:** autism; ASD; erythrocyte; multi-elements; toxic metals; essential minerals; rare earth elements; CARS

## Introduction

Autism spectrum disorder (ASD) is a lifelong neurodevelopmental disorder manifested by persistent deficits in social communication and social interaction, along with restricted and repetitive patterns of behavior, narrow interests and/or activities (APA 2013). The prevalence of autism estimated from European studies showed an increasing trend: 4.4 in 10,000 in the late 1960s and the early 1970s, 7.7 in the 1980s, and 9.6 in the 1990s [Gillberg & Wing, 1999]. There was also an upward trend in the prevalence of ASD in the United States in recent years. According to the Central Disease Control (CDC) (2014) of the United States, about 147 in 10,000 8-year-old children were diagnosed as ASD, which was roughly 30% higher than the previous estimate reported in 2012 (113 per 10,000) [Centers for Disease Control and Prevention (CDC), 2014]. This increased prevalence has enormous impacts on public health and has stimulated extensive research to understand the pathogenesis of ASD. Although there is not an officially reported prevalence available, the upward trend in the prevalence of ASD in China has received more and more attention.

The human body is composed of multiple chemical elements listed in the Periodic Table, and the human brain which is a specialized organ with a higher level of metals constitutes about 2% of body weight but receives 20% of blood supply [Bush, 2000; El-Ansary, Al-Daihan, Al-Dbass, & Al-Ayadhi, 2010]. The maintenance of ionic

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equilibrium is important for the normal brain functions. It is generally accepted that a higher body burden of heavy metals such as lead (Pb), cadmium (Cd), and mercury (Hg) and deficiencies in essential minerals such as zinc (Zn), magnesium (Mg), and iodine (I) can contribute to the development of ASD [Sullivan, 2009; Hamza, Hewedi, & Sallam, 2013; Yasuda, Yasuda, & Tsutsui, 2013; Rossignol, Genuis, & Frye, 2014; Yassa, 2014]. Moreover, results of several studies have shown that abnormal level of ions such as $Na^+$, $Ca^{2+}$, and $K^+$ in blood may play a role in the oxidative stress and the energy metabolism, may also contribute to the development of autism [Krey & Dolmetsch, 2007; El-Ansary et al., 2010]. Although autism has commonly been described as a brain-based disorder, several lines of evidence suggested that ASD may arise from systemic physiological abnormalities rather than central nervous system disorder [Herbert, 2005]. A recent reviewed study suggested that the etiology of ASD involves complex interactions between genetic factors and environmental toxicants that may act synergistically or in parallel during critical periods of neurodevelopment [Rossignol et al., 2014]. Therefore, abnormal contents of some chemical elements in the body were of vital importance of the neurodevelopmental disorders.

In general, the content of toxic metals and essential minerals of the human body can be determined by their presence in hair, nail, teeth, urine, and blood samples. Hair is more frequently used for determination of metals than other tissues since the collection of samples is a noninvasive procedure and it is readily available. In addition, hair is rich in several minerals. However, the reliability of quantitative analysis of minerals in hair remains debatable when compared with some other tissues such as blood [Namkoong, Hong, Kim, & Park, 2013]. After careful analysis and comparison, blood or blood fraction is widely recognized as a primary bioindicator representing the current element status in the body. The contents of many elements in erythrocytes could also be utilized as a useful biomarker of mineral status, since the majority of blood contents of several elements are localized in the erythrocytes [Nève, Molle, Hanocq, Sinet, & Geffel, 1983]. A recent study measured the concentrations of nine essential minerals in the red blood cell (RBC) and showed that the levels of selenium (Se) and molybdenum (Mo) in children with autism were significantly different from that in controls [Jory & Woody, 2008]. Several toxic elements such as Pb and Hg were measured in erythrocytes of children with autism [Geier, Audhya, Kern, & Geier, 2010; Adams et al., 2013]. However, most of the previous studies regarding the relationship of elements and ASD are less comprehensive and only limited to about 20 metals with little knowledge about the levels of other toxic metals and essential minerals in erythrocytes of autistic children. In addition, quantitative analysis has not been done to explore the correlation between the levels of abnormality in element concentration in erythrocytes and the severity of autistic syndrome.

The aim of the present work was to investigate the profile of multiple chemical elements including toxic metals, essential minerals and rare earth elements (REEs) in the erythrocytes of autistic versus typically developing children (TDC) of China. Qualitative and quantitative analyses were carried out to explore the possible correlation between levels of specific elements and the risk of ASD as well as the severity of the autistic symptom.

## Materials and Methods

### *Study Participants*

A total of fifty children with autism (including 42 boys and 8 girls), in a specific age group ranging from 2 to 8, were enrolled in the autism rehabilitation institutions in Beijing, China, and were included in the study. The following criteria were the requirements for the participants: scored 30 or more points in the Childhood Autism Rating Scale (CARS) [Schopler, Reichler, & Renner, 1988]. The clinical diagnosis of autistic children was performed by the experienced psychiatrists. As a control group, 50 typically developing individuals, aged 2–8 years, were recruited from kindergartens in Beijing. Also, the control participants were unrelated to a person with autism. An attempt was made to match the ages and sexes as closely as possible. Table 1 summarized the demographic characteristics of the autistic and TDC. The present study was approved by the Ethics Committee for Human Studies at Peking University Health Science Center (Permit Number: IRB00001052–13064). Participation was voluntary, and caregivers of all the participants read and signed informed consent forms before data collection.

CARS is a behavior-rating scale designed to identify children with autism as well as to quantitatively describe the severity of the disorder [Schopler et al., 1988]. It consists of 15 items including relating to people, imitation, emotional response, body use, object use, adaptation to change, visual response, auditory response, taste, smell and touch responses, fear or nervousness, verbal communication, nonverbal communication, activity level, level and consistency of intellectual response, and general impressions. Each item is scored from 1 (no symptom) to 4 (severe autistic symptoms). The CARS total score varies from 15 to 60 and the cut-off point for the diagnosis of autism is $\geq 30$. The autistic individual is considered mild-to-moderately autistic and severely autistic when his/her total score falls in the range of 30–36 and 37–60, respectively.

**Table 1. Demographic Characteristic of Autistic and Control Group**

| Characteristic | Autistic *N* (%) | Control *N* (%) | *P* value |
|---|---|---|---|
| CARS total scores | | - | |
| 30–36 (Mild-to-moderate) | 18 | - | |
| 37-60 (severe) | 32 | | |
| Gender | | | 0.227 |
| Male | 42 (84%) | 38 (76%) | |
| Female | 8 (16%) | 12 (24%) | |
| Age | | | 0.066 |
| 2–5 years | 38 (76%) | 30 (60%) | |
| 6–8 years | 12 (24%) | 20 (40%) | |
| Ethnic group | | | 0.371 |
| The Han | 43 (91.5%) | 42 (95.5%) | |
| The other ethnic | 4 (8.5%) | 2 (4.5%) | |
| Siblings | | | 0.499 |
| Yes | 12 (25.5%) | 10 (23.3%) | |
| No | 35 (74.5%) | 33 (76.7%) | |
| Birth weight, mean±SD (*N*), kg | 3.48 ± 0.61 (46) | 3.52 ± 0.62 (40) | 0.806 |
| Birth order, mean ± SD (*N*) | 1.16 ± 0.37 (44) | 1.20 ± 0.56 (41) | 0.330 |

*Measurement of Chemical Elements Concentration in RBC*

***Reagents.*** All the reagents such as nitric acids (Ultra Pure, UP) and peroxide (UP) were obtained from commercial resources. Standard solutions used for concentration determination were purchased from National Research Center for Certified Reference Materials (Beijing, PR China). De-ionized water (18 MΩ cm) was utilized for all analytical work. All labware were immersed in ~50% $HNO_3$ solution for at least 12 hr, and rinsed 20 times with de-ionized water before use.

***Sample preparation.*** In order to examine element levels in RBC, blood from the antecubital veins of subjects was collected and stored in EDTA tubes. Had the blood being drawn (approximately 30 min), the tubes were centrifuged at 2000 rpm for 10 min. The plasma and the white blood cell layers were removed and the packed RBCs were stored at −20°C for elemental analysis.

The UltraWAVE microwave digestion system (Milestone Inc., Italy) with single reaction chamber technology was used for sample digestion process. About 0.5 g of RBC samples were weighted out into quartz vessels followed by digestion with a mixture of $HNO_3$ and $H_2O_2$ in a volume ratio of 4:1. After the reaction chamber was pre-filled with nitrogen gas (~40.0 bar), the microwave system was programmed by giving gradual temperature rise steps of room temperature—150°C, 150–190°C, and 190°C-190°C for 5, 5, and 15 min, respectively. The resulting solution after microwave digestion was diluted to 15 mL with de-ionized water. The sample blank containing only acid mixture was prepared by the same method.

***Determination of metals.*** The metal measurements were carried out with an ICP-MS (Perkin Elmer ELAN DRC II, USA), an ICP-AES (Thermo iCAP 6000, Great Britain) and a Mercury Vapourmeter (DMA-80, Milestone Inc., Italy). These three instruments are housed in a clean lab with HEPA Class 1000-filtered air at the Medical and Pharmaceutical Analysis Center at Peking University.

The macro elements including Na, K, Ca, Mg, phosphorus (P), and S, and the trace elements with a higher level such as Zn and iron (Fe) were determined by ICP-AES. The remaining 26 trace elements measurements were performed by ICP-MS. These 26 trace elements are as follows: boron (B), titanium (Ti), V, chromium (Cr), manganese (Mn), cobalt (Co), copper (Cu), germanium (Ge), arsenic (As), Se, Rb, Sr, Mo, silver (Ag), Cd, Sb, I, cesium (Cs), Ba, La, Ce, Eu, Gd, Tl and Pb. V, Cr and Mn were determined by dynamic reaction cell (DRC) using $NH_3$ at a flow 0.6 mL/min. The instrumental settings for ICP-MS and ICP-AES were described in detail in an earlier publication [Li et al., 2011]. The measurement of Hg was set at 200°C for 3 min in the drying step followed by a decomposition step which was set at 650°C for 2 min by Mercury Vapourmeter.

The instruments were calibrated with the prepared working standard solution before the determination of samples. In addition, to check the instrument drift during operations, any one of the working standard solutions was carried out with each set of 15 samples. Sample blank was measured, and necessary correction was made during the determination of concentrations of various metal elements. The method accuracy was determined by the certified reference material whole blood (*ClinChek*® Whole Blood Control Level I order no: 8840, Recipe, Germany). The Limits of Quantification (LOQ) for 34 detected elements were shown in Table 2.

*Statistical Analyses*

The Statistical Package for Social Sciences (SPSS) software (Version 20.0, IBM) and R software were utilized for all statistical analysis, and two-tailed *P* values were calculated. Then the *P* values were adjusted via the Benjamini and Hochberg procedures to control the false discovery rate (FDR) [Benjamini & Hochberg, 1995; Benjamini & Hochberg, 2000]. The researchers set the threshold of the adjusted *P* values such that the expected number of false discoveries is less than 1. Independent samples *t*-test was used when means of metal levels from two groups were being compared. Comparisons among multiple groups were analyzed with one-way analysis of variance test. The nonparametric Mann-Whitney *U* test and Kruskal-Wallis rank sum test were used for comparisons of the non-normally distributed data. Chi-square test was applied

**Table 2. The Limits of Quantification (LOQ) for Detected Chemical Elements**

| Chemical elements | LOQ |
|---|---|
| Ag* | 257.40 |
| As# | 2.14 |
| B# | 14.81 |
| Ba* | 658.80 |
| Ca# | 1.15 |
| Cd* | 410.10 |
| Ce* | 87.60 |
| Co* | 382.50 |
| Cr* | 4860 |
| Cs* | 19.80 |
| Cu* | 540.90 |
| Eu* | 26.40 |
| Fe# | 1.74 |
| Gd* | 79.80 |
| Ge* | 800.10 |
| Hg* | 197.40 |
| I# | 22.52 |
| K# | 41.98 |
| La* | 28.20 |
| Mg# | 0.91 |
| Mn* | 325.00 |
| Mo* | 330.30 |
| Na# | 12.7 |
| P# | 5.81 |
| Pb* | 158.70 |
| Rb* | 129.00 |
| S# | 12.14 |
| Sb* | 245.40 |
| Se# | 23.95 |
| Sr* | 270.30 |
| Ti# | 5.50 |
| Tl* | 20.40 |
| V# | 2.37 |
| Zn# | 4.02 |

*Note*. * pg/mL, # ng/mL.

to evaluate the statistical differences among proportions of categorical variables.

## Results

*Characteristics of Participants*

A total of 100 Chinese children aged 2–8 were participated into this study including 50 children with ASD and 50 children with typical development. The demographic characteristics of all participants are shown in Table 1. The autistic and TDC groups were comparable with regard to gender and other key variables. No statistically significant difference was found in birth weight or birth order between the two groups.

*Multiple Chemical Elements Profile in Erythrocytes of Autism and Controls*

The median concentrations of 34 chemical elements in the erythrocytes of autistic children normalized to the median level in control children are given in Figure 1. The ratio value was ranked from the lowest to the highest. The levels of Pb and Cd were significantly higher than that in the control group. On the other hand, more elements were significantly lower in autistic children including Ba, La, Tl, Sb, V, Sr, Ca, Na, and Rb.

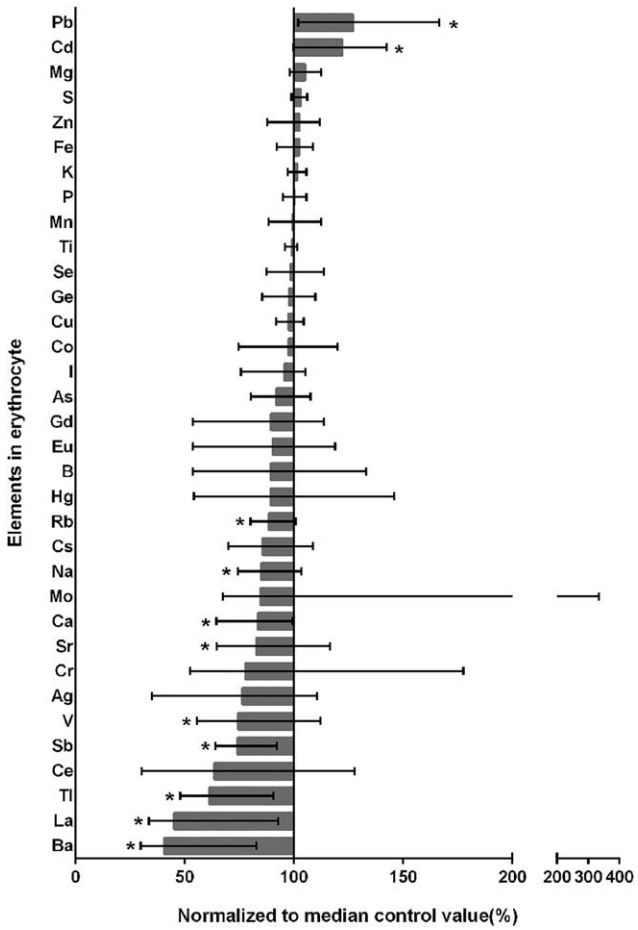


**Figure 1.** The median concentration of the autistic group normalized to the median value of control group. The ratio of median values is shown in columns, rescaled to the median control value as 100%. The horizontal bars display the 25th and 75th percentiles. Statistical significances between autistic and control groups are denoted by *. The original *P* values were adjusted via the Benjamini & Hochberg procedures to control the false discovery rate (FDR). We set the threshold of the adjusted *P* values such that the expected number of false discoveries is less than 1.

*Correlations between the Elements Levels in Erythrocytes and the CARS Total- and Sub-Scores*

Correlation analysis was carried out in order to try to understand the relationship between the changes in the level of chemical elements in the erythrocytes and the severity of autistic symptoms. In the present study, the total score of CARS in 50 autistic children varies from

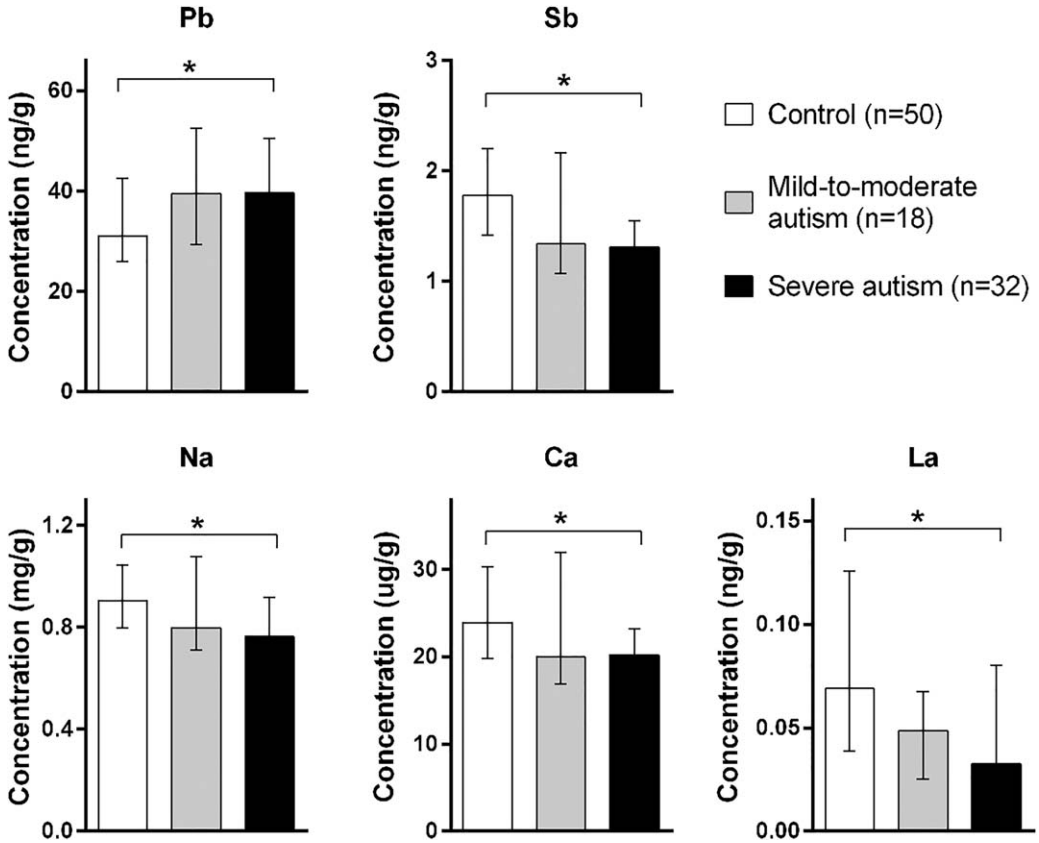


**Figure 2.** Relationship between CARS total scores and metal levels in erythrocytes of control and autistic subgroups. The median values of metals are shown in columns, and the vertical bars display the 25th and 75th percentiles. Statistically significances are denoted by *. The original *P* values were adjusted via the Benjamini & Hochberg procedures to control the false discovery rate (FDR). We set the threshold of the adjusted *P* values such that the expected number of false discoveries is less than 1.

34 to 49. Based on the severity of the symptom the children in the autistic group were divided into mid-to-moderately autistic ($n = 18$) and severely autistic ($n = 32$). Correlation between CARS total scores and metal levels in erythrocytes of control and autistic subgroups were analyzed and the results were presented in Figure 2. The content changes in five elements including Pb, Na, Ca, Sb, and La are associated with the CARS total scores. The researchers also performed multiple stepwise linear regression analysis to verify the correlations between the elements levels and the CARS total scores. Since the control group did not have the CARS scores, the researchers only used the data in autistic group to see the relationship between the CARS scores and the element levels. The final stepwise regression summary was reported in Table 3, from which it can be seen that a few elements are significantly correlated with the CARS total score. Note that the findings using the linear regression analysis are a little different from the previous findings shown in Figure 2. This is because here more assumption was used such as linear models when modeling the relationship between the levels of elements and the CARS total scores. Also findings in Figure 2 are based on adjusted *P* values because of multiple comparisons and consequently the findings are more conservative. The results from the multiple stepwise linear regressions could be seen as a supplementary proof for the correlations between elements levels and the CARS total scores.

Relationship between CARS scores of each subscale and metal levels in erythrocytes of control and autistic subgroups are shown in Figures 3–5. According to the subscale score of auditory response, the children in the autistic group is rated on a 1 to 3 point scale with $n = 18$, 19, and 13, respectively (Fig. 3). According to the subscale score of taste, smell, and touch response, the autistic group is rated on a scale of 1 to 3 point scale with $n = 34$, 11, and 5, respectively (Fig. 4). According to the subscale score of fear or nervousness, the autistic group is rated on a 1 to 3 point scale with $n = 23$, 21, and 6, respectively (Fig. 5). It could be easily observed that the abnormal levels of the listed metals in autistic group are correlated with the CARS scores of three subscales, including auditory response, taste, smell, and touch response as well as fear or nervousness.

## Discussion

Although trace elements in vivo constitute only less than 0.01% of body weight, they have been shown to play a major role in the normal function of the central nervous system [Fido, 2005]. However, little is known about the multiple chemical elements profile in specific biological samples such as erythrocytes. In the present study, 34 elements including REEs in erythrocytes of autistic and TDC were examined for the first time.

**Table 3. Final Summary of Stepwise Regression Analysis of Relationship between CARS Total Scores and Element Levels in Erythrocytes of Autistic Subgroups**

| | Coefficients | | | |
|---|---|---|---|---|
| | Estimate | Std. Error | *t* value | Pr(>\|t\|) |
| (Intercept) | 7.354e+00 | 1.781e+01 | 0.413 | 0.6829 |
| As | −5.894e−01 | 2.726e−01 | −2.162 | 0.0393 * |
| B | −1.066e−01 | 8.006e−02 | −1.331 | 0.1939 |
| Ca | −5.156e−01 | 2.674e−01 | −1.928 | 0.0640 † |
| Cd | 1.247e+01 | 4.645e+00 | 2.685 | 0.0120 * |
| Co | −2.415e+01 | 7.984e+00 | −3.025 | 0.0053** |
| Cs | 8.422e−01 | 7.163e−01 | 1.176 | 0.2496 |
| Cu | 1.734e−02 | 9.863e−03 | 1.758 | 0.0896 † |
| Eu | −3.445e+02 | 1.942e+02 | −1.774 | 0.08700 † |
| Fe | 4.736e−02 | 1.723e−02 | 2.749 | 0.0103 * |
| Gd | −1.006e+02 | 5.254e+01 | −1.916 | 0.0657 † |
| Ge | −1.359e+01 | 5.646e+00 | −2.406 | 0.02300 * |
| Hg | −5.022e−01 | 2.365e−01 | −2.124 | 0.0426 * |
| Mn | 1.614e−01 | 1.179e−01 | 1.369 | 0.1819 |
| Na | 1.231e−02 | 9.839e−03 | 1.252 | 0.2211 |
| Pb | −3.095e−02 | 2.693e−02 | −1.149 | 0.2603 |
| Rb | −1.925e−03 | 1.532e−03 | −1.257 | 0.2193 |
| Sb | 2.302e+00 | 2.022e+00 | 1.138 | 0.2647 |
| Sr | 3.216e−01 | 2.074e−01 | 1.551 | 0.1323 |
| Tl | 6.313e+01 | 4.299e+01 | 1.468 | 0.1532 |
| V | 2.742e+00 | 9.633e−01 | 2.846 | 0.0082 ** |
| Zn | −9.039e−04 | 6.532e−04 | −1.384 | 0.1774 |

*Note*. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '†' 0.1 ' ' 1.
Residual standard error: 3.365 on 28 degrees of freedom.
Multiple R-squared: 0.5753, Adjusted R-squared: 0.2567.
F-statistic: 1.806 on 21 and 28 DF, *P* value: 0.07182.

### *Changes of Element Levels in Erythrocytes of Children with ASD*

As shown in Figure 1, levels of 11 out of 34 elements (32%) of the autistic group were significantly different from those of the control group.

Mercury and lead were mostly studied heavy metals previously [Rossignol et al., 2014]. Bernard, Enayati, Redwood, Roger, and Binstock [2001] pointed out that the exposure to thimerosal which was normally used as preservatives may lead to an increased risk of autism. However, a recent study provided a contradictory result [Taylor, Swerdfeger, & Eslick, 2014]. In the eight studies comparing blood mercury level between autistic and TDC, three studies reported an elevated level [Ip, Wong, Ho, Lee, & Wong, 2004; Geier et al., 2010; Yassa, 2014], while the other five concluded as no difference [Hertz-Picciotto et al., 2010; Stamova et al., 2011; Albizzati, Morè, Di, Saccani, & Lenti, 2012; Adams et al., 2013; Rahbar et al., 2013]. In the present study, no significant differences were found in the levels of mercury in erythrocytes between children with ASD and without. However, it is worthwhile to note that there were several extremely high values (3–5 times higher than the control levels) for mercury in children with autism. Therefore, it cannot be excluded that high burden of mercury may be an important environmental factor in contribution to the etiology of autism, at least in some individuals, since the disorder is a spectrum and is expected to have different causes.

Lead is a confirmed toxic element that can affect several organ systems including the nervous, hematopoietic,

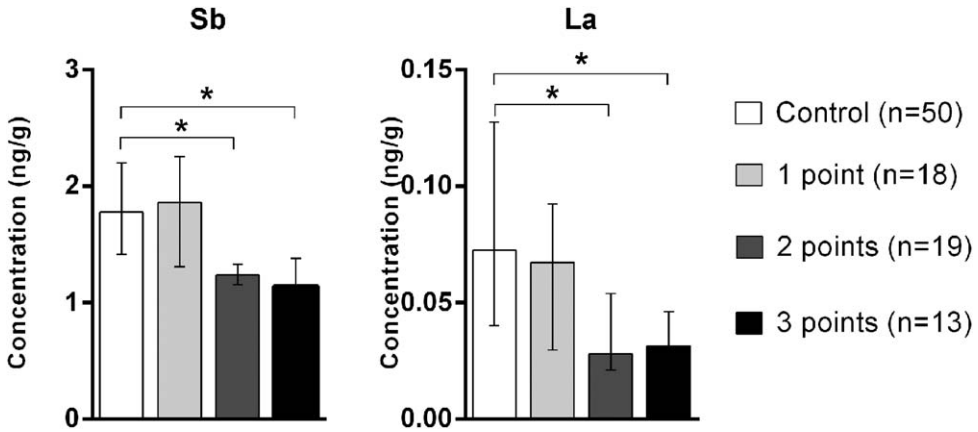


**Figure 3.** Relationship between CARS subscores of auditory response and metal levels in erythrocytes of control and autistic subgroups. The median values of metals are shown in columns, and the vertical bars display the 25th and 75th percentiles. Statistical significances are denoted by *. The original *P* values were adjusted via the Benjamini & Hochberg procedures to control the false discovery rate (FDR). We set the threshold of the adjusted *P* values such that the expected number of false discoveries is less than 1.

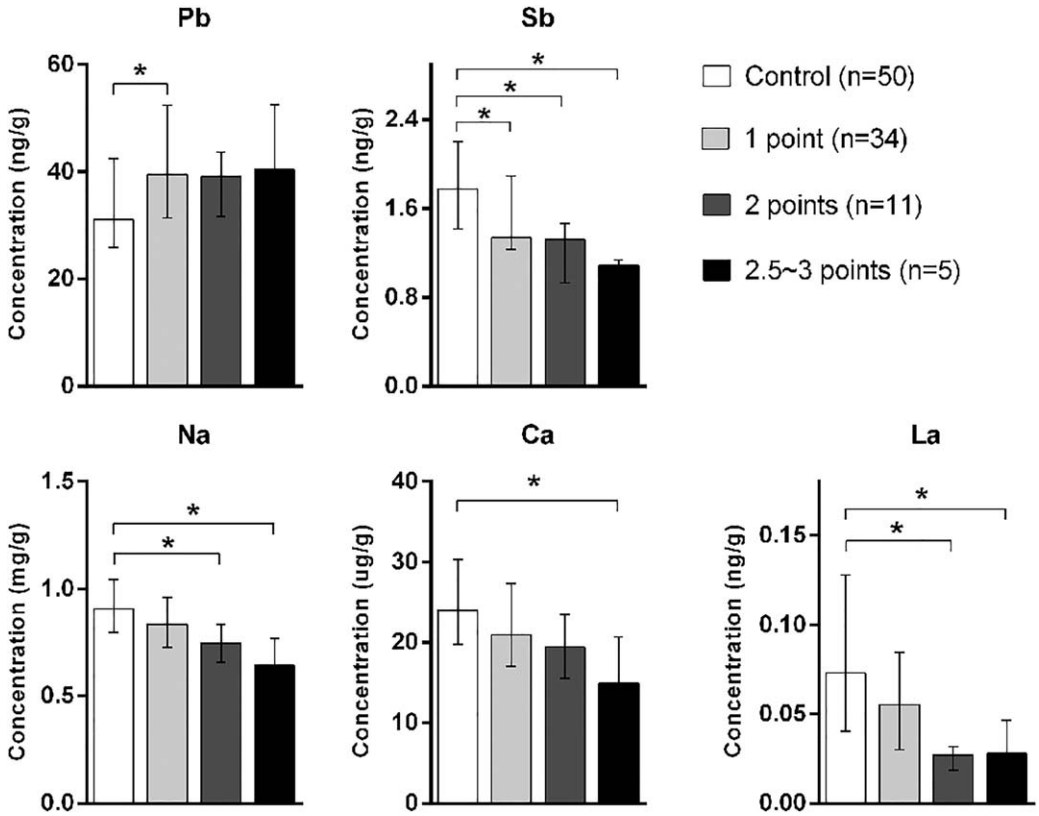


**Figure 4.** Relationship between CARS subscores of taste, smell and touch response and metal levels in erythrocytes of control and autistic subgroups. The median values of metals are shown in columns, and the vertical bars display the 25th and 75th percentiles. Statistical significances are denoted by *. The original *P* values were adjusted via the Benjamini & Hochberg procedures to control the false discovery rate (FDR). We set the threshold of the adjusted *P* values such that the expected number of false discoveries is less than 1.

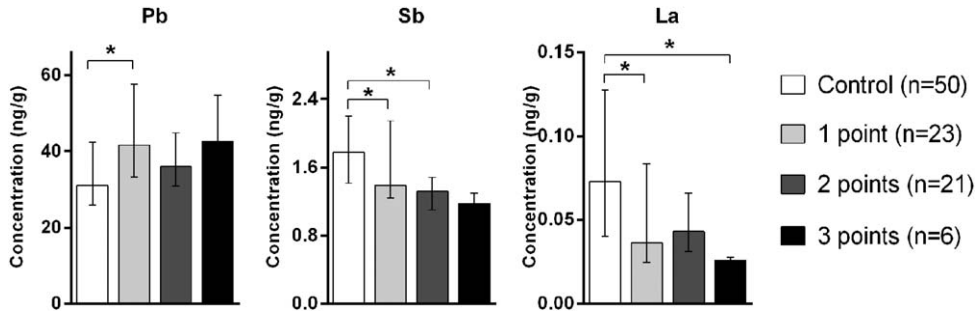


**Figure 5.** Relationship between CARS subscores of fear or nervousness and metal levels in erythrocytes of control and autistic subgroups. The median values of metals are shown in columns, and the vertical bars display the 25th and 75th percentiles. Statistical significances are denoted by *. The original *P* values were adjusted via the Benjamini & Hochberg procedures to control the false discovery rate (FDR). We set the threshold of the adjusted *P* values such that the expected number of false discoveries is less than 1.

renal, endocrine, and skeletal [Goyer, 1997]. The toxic effects of lead on cognitive and behavioral development in infants and young children attracted most attention [National Research Council (US) Committee on Measuring Lead in Critical Populations, 1993]. In the ten studies comparing blood levels of lead between normal and ASD groups, five studies reported an elevated level [Cohen, Johnson, & Caparulo, 1976; El-Ansary et al., 2010; El-Ansary, Bacha, & Al-Ayadhi, 2011; Adams et al., 2013; Yassa, 2014], while the other five reported no difference [Cohen, Paul, Anderson, & Harcherik, 1982; Laura et al., 2011; Tian et al., 2011; Albizzati et al., 2012; Rahbar

et al., 2015]. In the current study we found a significantly higher level of lead in autistic children (+27%, adjusted $P$ value = 0.018), which ranked the highest among all the elements studied (Fig. 1). However, only two studies of these ten examined the level of lead in RBC [El-Ansary et al., 2011; Adams et al., 2013]. The inconsistency regarding the changes of lead content in ASD may reflect various causes of ASD in the different population studied as well as different tissues used in these studies.

In addition, it was shown in this study that the cadmium level in erythrocytes was significantly detected in autistic children (+22%, adjusted $P$ value = 0.051). It seemed to be in line with the finding of Laura et al. [2011] who reported that the level of Cd in blood was significantly increased in autistic children compared to control groups.

For the essential minerals, previous studies carried out on the chemical elements in hair documented that children diagnosed with autism were deficient in iodine, phosphorus, lithium, zinc, magnesium, and calcium [Adams, Holloway, George, & Quig, 2006; Blaurock-Busch, Amin, & Rabah, 2011; Yasuda et al., 2013]. However, there are few published reports concerning the essential minerals level in erythrocytes of ASD. These findings have stimulated our interest to explore the potential changes in levels of essential minerals including six macro elements and some micro elements in the erythrocytes. The result indicated a significant deficiency in some essential minerals, including Ca (−16%, adjusted $P$ value = 0.025), Na (−15%, adjusted $P$ value = 0.018) and Sr (−17%, adjusted $P$ value = 0.043). Furthermore, this study examined the levels of REEs (La, Ce, Gd, and Eu) in erythrocytes of children with and without ASD for the first time. As displayed in Figure 1, the concentration of La had a dramatic decrease compared to the control level (−55%, adjusted $P$ value = 0.0013). The concentrations of other three REEs (Ce, Gd, and Eu) were in the same range as that of the control group.

The above results indicated that autistic children were suffering from element imbalances, which suggested that the abnormal levels of chemical elements in erythrocytes might have a catastrophic impact on neuronal functions.

*Correlation between Chemical Elemental Levels and Clinical Manifestations*

It was shown that 11 elements in children with ASD were significantly different from control group. Five out of the 11 elements were found to be correlated to the degrees of the severity of autistic syndrome in terms of total score of CARS (Fig. 2), suggesting that these five elements may have significant impact on ASD severity. Moreover, changes in the levels of certain metals are correlated to scores of particular subscales. The correlation between levels of toxic metal and the severity of autistic symptoms had also been reported by other studies. Lakshmi and Geetha [2011] reported that a significant elevation in Pb concentration in the hair and nail of ASD was well correlated with the degrees of ASD severity.

Furthermore, the decrease of essential elements including Na and Ca was associated with the impairment of taste, smell, and touch (Fig. 4). The result indicated that the deficiency of essential elements contributed to the increased risk of ASD and had a strong correlation with the severity of the autistic symptom. Several studies have reported that children with ASD have significantly more feeding problems and eat a narrower range of foods than TDC, which may be a result of sensory sensitivity [Schreck & Williams, 2006; Cermak, Curtin, & Bandini, 2010]. Therefore, it is unclear whether the sensory sensitivity leads to the nutritional insufficiency or the nutritional insufficiency leads to the sensory sensitivity. There seems to be an intrinsic connection between nutritional insufficiency and sensory sensitivity which might interact as both cause and effect.

In addition, it is worthwhile to note that the degree of the decrease of Sb and La was not only associated with the severity of the autistic syndrome as a whole (Fig. 2), but also specifically with the impairment in three aspects: auditory response (Fig. 3), taste, smell, and touch (Fig. 4), and fear or nervousness (Fig. 5). To our knowledge, these results have not been reported so far, and their underlying biological and genetic basis is unknown. Further study and verification are needed.

*The Possible Role of Metals in the Development of ASD*

It is generally accepted that ASD is a complex disorder resulting from the combination of genetic and environmental factors [Chaste & Leboyer, 2012; Rossignol et al., 2014]. Results of many studies indicated that autistic children may not possess sufficient metabolic reserve to detoxify environmental pollutants and/or heavy metals as efficiently as TDC due to differences in genetics and may therefore be more susceptible to environmental toxics [James et al., 2006; Deth, Muratore, Benzecry, Power-Charnitsky, & Waly, 2008; Stamova et al., 2011; Tian et al., 2011]. Interesting results from a recent study showed that there are differences in uptake of multiple toxic and essential elements over the second and third trimesters and early postnatal periods in monozygotic (MZ) and dizygotic (DZ) twins discordant for ASD, which brought to a shocking change of perspective in the field of metal dysregulation in ASD [Arora et al., 2017]. For the reasons not fully understand, evidence from many studies indicated that

abnormal levels of specific chemical elements may contribute to the development of ASD [Krey & Dolmetsch, 2007; Sullivan, 2009; El-Ansary et al., 2010; Hamza et al., 2013; Yasuda et al., 2013; Rossignol et al., 2014; Yassa, 2014]. To date it is not absolutely clear what are the roles of the abnormal tissue concentrations of many elements in the pathogenesis of ASD. As suggested by Strozyk and Bush [2006], "abnormalities of metal ion affecting the biochemistry in neural tissue arise by two basic mechanisms, protein aggregation mediated by metal ions and oxidative reactions catalyzed by redox-active metals."

It was proved that lead exposure to children were associated with cognitive impairment, attention deficits, learning and behavioral disabilities, immune and sensory dysfunctions [Goyer, 1993; Cory-Slechta, 1995; Brockel & Cory-Slechta, 1998; Lanphear et al., 2005]. Jakubowski reported that an elevated blood lead was associated with reduced Intelligence Quotient (IQ) [Jakubowski, 2011]. Moreover, neurodevelopmental deficits of children were reported to occur at very low levels of lead exposure (below 5 $\mu$g/dL) and there was so far no safe threshold for lead exposure to children [Min et al., 2007].

The mechanisms of the toxic effects of lead on nervous system have been reported to be manifold. Lead induced pathological changes in motor axons include segmental demyelination and axonal degeneration [Landrigan, 1989]. As a divalent cation, lead has been known to mimic some biological actions of calcium [Ngueta, Gonthier, & Levallois, 2015]. Lead can disrupt transmitter release by its high-affinity interactions with the voltage-gated calcium channels and calcium-activated proteins. The affinity of lead to these proteins are about 100,000 times higher than that of calcium preventing the normal function of these proteins [National Research Council (US) Committee on Measuring Lead in Critical Populations, 1993; Suszkiw, 2004]. In addition, lead may cause adverse effects through inhibition of the glutamine synthetase, which resulted in disturbed brain energy metabolism. This made the brain more vulnerable to oxidative stress, since there are plenty of polyunsaturated fatty acids in its membranes, serving as targets of lipid peroxidation (LPO) [El-Ansary et al., 2011]. Oxidative stress has been shown to have significant impact on blood–brain barrier (BBB) [Heo, Han, & Lee, 2005]. Thus lead is able to induce more serious neurotoxicity by its high level passage through the damaged BBB. Lead has also been reported to regulate the expression of many genes associated with immunological and inflammatory processes, and a dysregulation of the immune response was proved to be associated with autism [Tian et al., 2011].

Cadmium is also a serious environmental toxicant being placed in the list of top 20 hazardous substances [Agnihotri et al., 2015]. It is nutritionally nonessential, yet can alter the antioxidant defense system and induce oxidative stress in the body. Many studies indicated that reactive oxygen species (ROS) could be generated by Cd exposure, which leads to injury of proteins and DNA, inflammation, tissue damage, and subsequent cellular apoptosis [Uttara, Singh, Zamboni, & Mahajan, 2009]. Furthermore, Cd toxicity affected the metabolism of essential metals such as calcium, zinc, and iron. Calcium, in particular, played a key role in maintaining energy metabolism [Goyer, 1997].

The above mentioned three essential minerals including Na, Ca, and Sr are known to be closely related to oxidative stress and energy metabolism. According to Crompton, Künzi, and Carafoli [1977], both $Na^+$ and $Sr^{2+}$ were able to initiate an exchange with $Ca^{2+}$ from respiring mitochondria. When intracellular calcium increases, mitochondrial oxygen radical production can be stimulated [El-Ansary et al., 2010]. As a result, the function of mitochondria is affected, which plays an important part in neurodegenerative diseases. Apoptosis cascade can be triggered resulting in cell death since mitochondria is known to be heavily involved in ATP synthesis, free oxygen radical production and apoptogenic factor release into the cytosol [Castaldo et al., 2009]. El-Ansary et al. [2010] demonstrated that the decrease of plasma calcium level could be attributed to the increase of cytosolic $Ca^{2+}$ in brain cells.

It has been reported that calcium deficiency could elevate blood lead accumulation and potentially increase the susceptibility to lead toxicity [Aungst & Fung, 1985]. On the other hand, supplementation of the diet with nutritional elements such as calcium and zinc can decrease lead absorption in humans and experimental animals [Peraza, Ayala-Fierro, Barber, Casarez, & Rael, 1998]. Therefore, not only the imbalance of elements levels but also the interaction between chemical elements may be associated with neurodevelopmental disorders.

The rare earth elements (REEs) are identified as a chemically uniform group which has similar physiochemical characters [Ramos et al., 2016]. Although, the environmental contamination and exposure caused by applications and mining of the mineral of REEs has improved risks of human health [Hirano & Suzuki, 1996], it should be noted that REEs, like a number of other xenobiotics, follow hormetic concentration-related trends, implying stimulatory or protective effects at low levels, then adverse effects at higher concentrations [Pagano, Guida, Tommasi, & Oral, 2015]. In addition, many studies have reported on antioxidant effects of some REEs suggesting ad hoc clinical applications [Schubert, Dargusch, Raitano, & Chan, 2006; Wong & McGinnis, 2014].

In this study, decrease in REEs level seemed to be correlated with the severity of autistic syndrome. It can thus be hypothesized that when REEs level decreased, their stimulatory or protective effects declined in parallel which may contribute to the development of autism. This study examined the levels of REEs (La, Ce, Gd, and Eu) in erythrocytes of children with and without ASD for the first time, and the results have not been reported so far. More research is needed to explore the possible role of REEs in the development of ASD.

Collectively, this study corroborated data from previous studies indicating the presence of multiple element imbalances in the erythrocytes of autistic children. Even though knowledge of how elements attribute to autism is still at an elemental stage, the few available pieces of data suggest that it could be a promising area for further investigations.

*Limitation*

It is obvious that the results of the present study should be interpreted with caution. ASD is a heterogeneous disorder and can be divided into subtypes based on the characteristic behavioral manifestations (Wing et al.). It would be interesting to compare the differences in element contents among these subtypes to see if certain element profile correlates with specific behavioral pattern to elucidate the mechanisms underlying the disorder. The sample size in the present study provides enough power to compare the differences in element contents between the ASD and TDC groups but does not allow comparisons between subgroups of ASD.

There are several tools available for ASD diagnosis and severity evaluation. Only CARS was employed in the present study. Other tools such as ADOS and ADI-R were not used to assess the symptom. CARS is designed to be used by the parents or care takers of the patients. On the other hand, ADOS and ADI-R are tools used by health professionals with special license and might be viewed by some investigators as more rigorous tools in diagnosis of ASD.

Lastly, all the participants of the study were recruited from Beijing area. Given the substantial contribution of environmental factors for the pathogenesis of ASD [Hallmayer et al., 2011], patients recruited from various geographical areas should be considered in the future studies in order to understand the impact from various factors such as types of food on element profile and the symptoms of the disorder.

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## Conflict of interest

The authors declare they have no actual or potential competing financial interests.

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