Urinary aluminium and its association with autism spectrum disorder in urban preschool children in Malaysia

Mohd Shahrol Abd Wahil [1, 2] , **Mohd Hasni Ja'afar** [Corresp., 1] , **Zaleha Md Isa** [1]

[1] Department of Community Health, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Kuala Lumpur, Malaysia

[2] Sector of Occupational and Environmental Health, Disease Control Division, Ministry of Health Malaysia, Putrajaya, Putrajaya, Malaysia

Corresponding Author: Mohd Hasni Ja'afar
Email address: drmhasni@ukm.edu.my

Background The presence of aluminium (Al) in the human body may impact brain neurodevelopment and function, and it is thought to contribute to autism spectrum disease (ASD). The main objective of this study was to assess the association between urinary Al and the development of ASD among Malaysian preschool children in the urban city of Kuala Lumpur. **Method** This was an unmatched case–control study in which children with ASD were recruited from an autism early intervention center and typically developed (TD) children were recruited from government-run nurseries and preschools. Urine samples were collected at home, assembled temporarily at study locations, and transported to the laboratory within 24 hours. The Al concentration in the children's urine samples was determined using inductively coupled plasma mass spectrometry (ICP-MS). **Result** A total of 155 preschool children; 81 ASD children and 74 TD children, aged 3 to 6 years, were enlisted in the study. This study demonstrated that ASD children had significantly higher urinary Al levels than TD children (median (interquartile range (IQR): 2.89 (6.77) µg/dL versus 0.96 (2.95) µg/dL) ($p<0.001$). Higher parental education level, non-Malay ethnicity, male gender, and higher urinary Al level were the significant ASD risk factors (adjusted odds ratio (aOR)>1, $p<0.05$). **Conclusion** A higher urine Al level was discovered to be a significant risk factor for ASD among preschool children in the urban area of Kuala Lumpur, Malaysia.

1 **Urinary aluminium and its association with autism spectrum disorder in urban**
2 **preschool children in Malaysia**

3 **Mohd Shahrol Abd. Wahil [1,2], Mohd Hasni Ja'afar [1]*, Zaleha Md Isa [1]**

4 [1] Department of Community Health, Faculty of Medicine, National University of Malaysia,
5 Cheras, Kuala Lumpur, Malaysia
6 [2] Sector of Occupational and Environmental Health, Disease Control Division, Ministry
7 of Health Malaysia, Putrajaya, Malaysia

8 * Corresponding author
9 Name: Mohd Hasni Ja'afar
10 Address: Department of Community Health, Faculty of Medicine, National University
11 of Malaysia, Jalan Yaacob Latiff, Bandar Tun Razak, Cheras, 56000, Kuala Lumpur,
12 Malaysia
13 Email address: drmhasni@ukm.edu.my

Abstract

16 **Background** The presence of aluminium (Al) in the human body may impact brain
17 neurodevelopment and function, and it is thought to contribute to autism spectrum
18 disease (ASD). The main objective of this study was to assess the association between
19 urinary Al and the development of ASD among Malaysian preschool children in the urban
20 city of Kuala Lumpur.

21 **Method** This was an unmatched case–control study in which children with ASD were
22 recruited from an autism early intervention center and typically developed (TD) children
23 were recruited from government-run nurseries and preschools. Urine samples were
24 collected at home, assembled temporarily at study locations, and transported to the
25 laboratory within 24 hours. The Al concentration in the children's urine samples was
26 determined using inductively coupled plasma mass spectrometry (ICP-MS).

27   **Result** A total of 155 preschool children; 81 ASD children and 74 TD children, aged 3 to

28   6 years, were enlisted in the study. This study demonstrated that ASD children had

29   significantly higher urinary Al levels than TD children (median (interquartile range (IQR):

30   2.89 (6.77) μg/dL versus 0.96 (2.95) μg/dL) (p<0.001). Higher parental education level,

31   non-Malay ethnicity, male gender, and higher urinary Al level were the significant ASD

32   risk factors (adjusted odds ratio (aOR)>1, p<0.05).

33   **Conclusion** A higher urine Al level was discovered to be a significant risk factor for ASD

34   among preschool children in the urban area of Kuala Lumpur, Malaysia.

35   **Introduction**

36   Autism spectrum disorder (ASD) is an early-onset neurodevelopmental condition

37   characterized by altered social interaction and communication, as well as repetitive and

38   rigid behavior, together with restricted interests (APA 2013). It has the potential to

39   substantially affect major aspects of life and lower quality of life beginning in childhood

40   and continuing into later adulthood (Bölte et al. 2019). Individuals with ASD may

41   experience the aforementioned characteristics to variable degrees and demonstrate a

42   broad range of abilities, which can range from being extremely gifted to being severely

43   challenged (Freedman 2010).

44   ASD prevalence has significantly grown globally from its first description in 1943, when

45   there were 4.5 cases per 10,000 children (Kanner 1943). For instance, the prevalence of

46   ASD in the United States grew from 3.4 cases per 1000 children in the early 2000s

47   (Fombonne 2003) to 20.0 cases per 1000 children in 2011 (Blumberg et al. 2013). In the

48   United Kingdom, the prevalence of ASD in 2009 was 9.4 cases per 1000 children (Baron-

49   Cohen et al. 2009). While the prevalence of ASD in Malaysia in 2011 was 15.9 cases per

50   1000 children (Kaur et al. 2015).

51   Aluminium (Al) is the most ubiquitous metal and the third most abundant element in the

52   Earth's crust (Exley 2009). It has no known biological role in human and is not essential

53 at any quantity (Rahbar et al. 2015). However, its distinguishing qualities, such as
54 lightweight, strength, and ease of sterilization, make it suitable for use in pharmaceuticals,
55 water, food, and other household items (Corkins 2019). In view of its widespread usage,
56 humans are at high risk of exposure either through oral ingestion, skin absorption
57 (wounds), or occupational inhalation (ATSDR 2008). Once Al enters the circulation and
58 tissue fluids, it swiftly equilibrates with chemically similar elements in the body and
59 distributes broadly (Priest 1993). Only the skeleton contains a high concentration of Al,
60 accounting for 54.0% of the body's total Al content (Corkins 2019). Aside from skeleton,
61 one of the most important target tissues for Al accretion is the brain (Bondy 2016), which
62 may penetrate the blood-brain barrier (BBB) via particular carrier protein complexes
63 (Yokel et al. 2001; Yokel et al. 2002). Al is predominantly eliminated from the body through
64 urine and feces in humans (ATSDR 2008).

65 Al intoxication in humans has been shown to have impacts on the musculoskeletal
66 system, the hematological system, and the neurological system (Crisponi et al. 2013).
67 **Studies have shown that the neurotoxic effect of Al is caused by oxidative stress,**
68 **which leads to cell death (apoptosis) through; 1) interactions with transitional**
69 **metals like copper and iron** (Becaria et al. 2003; Di & Bi 2004), 2) inhibition of
70 cytochrome oxidase (in the case of Al phosphate (AlP) toxicity) (Chugh et al. 1993), 3)
71 increased lipid peroxidation (Ferretti et al. 2003; Verstraeten et al. 2008), 4) enhancer of
72 6-hydroxydopamine (Sánchez-Iglesias et al. 2009), and 5) generation of reactive oxygen
73 species (ROS) in electron transport chain (Campbell 2002; Kumar & Gill 2014).
74 Additionally, Al complexing changes several features of BBB permeability in such a way
75 that an Al-amyloid beta complex would be more readily penetrate to brain cells than
76 amyloid beta alone (Al-Ayadhi et al. 2012; Banks et al. 2006).

77 Children are considered a vulnerable population because they may be more susceptible
78 to health consequences than adults, and the link may vary depending on developmental
79 stage (Bearer 1995). Compared to adults, children might also have a different capacity
80 for repairing damage from neurotoxicant insults and have a longer remaining lifetime in
81 which neurotoxicant damage can be expressed (Rice & Barone Jr 2000). In addition, both

82 the prenatal and postnatal periods are crucial for anatomical and functional brain
83 development, in which exposure to neurotoxicant in these phases might impair the brain
84 more significantly (Gale et al. 2004). Furthermore, the health consequence that we are
85 most concerned about in children is that the harm may not appear until a later
86 developmental stage (WHO 2006).

87 According to past research, Al is linked to neuropathological abnormalities seen in
88 neurodegenerative illnesses in adults such as Alzheimer's disease, Parkinson's disease,
89 amyotrophic lateral sclerosis, and multiple sclerosis (Bondy & Campbell 2017).
90 Nonetheless, toxicological research on the effects of Al on neurodevelopmental disorders
91 in young children, particularly ASD, is scarce. Besides that, the statistical profile of Al in
92 humans, particularly children, in relation to ASD in Malaysia is unavailable. In view of the
93 foregoing, the primary goal of this study was to examine the relationship between urinary
94 Al and ASD among preschoolers in Kuala Lumpur, Malaysia.

95 **Material & Methods**

96 This was an unmatched case-control study involving 155 preschool children, consisting
97 of 81 ASD children and 74 TD children (age 3 to 6 years) in the urban city of Kuala Lumpur
98 in the year of 2019. The ASD kids were selected at random from the *Genius Kurnia* center
99 in Kuala Lumpur, which is a government center for early autism intervention. The TD
100 children were also randomly selected from *Tabika Kemas* public preschools and *Taska*
101 *Kemas* public nurseries in Kuala Lumpur. The study sites for case and control group were
102 also randomly selected to maintain the random effect in the study. The pediatrician's
103 diagnosis was according to Diagnostic and Statistical Manual of Mental Disorders fifth
104 edition (DSM-5) criteria (APA 2013) and the International Classification of Diseases tenth
105 revision (ICD-10) (WHO 2004). As per the Modified Checklist for Autism in Toddlers (M-
106 CHAT) assessment, the TD children were declared healthy by the pediatrician. For both
107 groups, the following exclusion standards are applied; 1) other neurobehavioral disorders,
108 2) congenital abnormality or syndrome, 3) acute infectious diseases, 4) recent surgical
109 procedures, 5) endocrine problems, and 6) on oral/infusion drugs (chemotherapy) or

110 chelation treatment for eliminating heavy metals. The selection of the respondents is
111 illustrated in Figure 1.

112 The evaluation included the participation of both the parents and the children. An online
113 questionnaire that the parents were supposed to fill out on their own time was sent to
114 them. The children were obliged to provide clean-catch urine in the morning. Urine
115 samples were obtained once by the parents at their residence and then handed over to
116 the investigator on the same day that their children started attending classes (at the
117 *Genius Kurnia* center, *Tabika Kemas* preschools, and *Taska Kemas* nurseries). The urine
118 samples were then transported within 24 hours to an environmental laboratory, located
119 40.0 km from the study locations at the National University of Malaysia (UKM), Bangi,
120 Selangor state. These samples were stored at -20.0°C before laboratory analysis. The
121 urine samples were then treated with a 0.2% nitric acid (HNO_3) solution to facilitate the
122 breakdown of organic particles in the urine sample (Abd Wahil et al. 2021), followed by
123 Al assay using inductively coupled plasma mass spectrometry (ICP-MS) on a
124 PerkinElmer SCIEX™ ELAN® 9000. The system was calibrated, and internal
125 standardization was conducted to ensure good performance of the system in term of
126 sensitivity and accuracy. The Al detection limit was from 1.0 to 10.0 parts per trillion (ppt).
127 The concentration readings of Al were obtained from computer software linked to the ICP-
128 MS machine. Finally, the ICP-MS results and questionnaire data set were analyzed using
129 the software of International Business Machines (IBM), Statistical Package for Social
130 Sciences (SPSS), version 22.

131 The normality of the data was examined graphically using Q-Q plot and histogram and
132 statistically by Kolmogorov-Smirnov statistics, kurtosis, skewness, mean and median.
133 Due to the non-normal distribution of the data, the non-parametric tests such as Mann-
134 Whitney U test and Kruskal-Wallis test were used to measure the comparison of Al levels
135 between groups. Multiple logistic regression was performed to assess the association
136 between urinary Al and the development of ASD among preschool children.

137   The Ministry of Health (MOH) Malaysia Medical Research and Ethics Committee (NMRR-

138   18-3765-45117) and the National University of Malaysia (UKM) Research and Ethics

139   Committee (FF-2018-286) approved the study in 2019. All processes were carried out in

140   accordance with the principles of the Helsinki Declaration of 1964 and its amendments.

141   Prior to the study, parents provided informed written agreement and participated

142   voluntarily.

143   **Results**

144   A total of 155 individuals were recruited for this study, with 81 ASD children in the case

145   group and 74 TD children in the control group. The demographic characteristics are

146   shown in Table 1. From this table, there was a significant difference in composition

147   between the two groups in terms of parental education level, family monthly income,

148   ethnicity, and children's gender. Most of the ASD children's parents had tertiary education

149   (85.2%), however, most of the TD children's parents had secondary education (60.8%)

150   (p<0.001). The ASD families had an average monthly income of RM 6699.94 (± RM

151   3856.52) while that of the TD families was RM 3960.03 (±RM 2025.19) (p<0.001). About

152   51.9% of the ASD families had a monthly income of >RM 5000, whereby, only 21.6% of

153   the TD families had a monthly income of >RM 5000 (p<0.001). Among the ASD and TD

154   children, 77.8% and 94.6% were Malay, respectively (p=0.003). Male gender was

155   predominant among the ASD children (84.0%) with male: female ratio of 5:1, while TD

156   group had an almost equal proportion of boys and girls (p<0.001). The average age of

157   the ASD and TD children was 5.63 ± 0.60 years and 5.45 ± 0.83 years, respectively

158   (p>0.05).

159   In Table 2, the concentration of urinary Al was compared between ASD children and TD

160   children. The ASD children were found to have a significantly higher median (interquartile

161   range (IQR)) urinary Al level than the TD children (2.89 (6.77) µg/dL versus 0.96 (2.95)

162   µg/dL) (p<0.001). Furthermore, the ASD children also had higher level of minimum,

163   maximum, mean (±SD), and geometric mean urinary Al than the TD children. When ASD

164 children were classified according to the severity of the disease, urinary Al levels were
165 surprisingly increased in a trend with the severity of the ASD (p<0.001) (Figure 2).

166 The receiver operating characteristic (ROC) curve analysis was performed to determine
167 the urinary Al level cut-off. The urinary Al area under the curve had a significant value of
168 0.75 and close to the value of 1.0 (Figure 3). The value of 1.0 shows that the model is
169 perfect. According to the Youden index, the cut-off level of urinary Al was 1.60 µg/dL,
170 higher than the standard reference of 0.58–0.77 µg/dL from the report of the Agency for
171 Toxic Substances and Disease Registry (ATSDR 2004). This new cut-off level of urinary
172 Al was used to categorize urinary Al into 2 groups (> 1.6 µg/dL and ≤ 1.6 µg/dL) in the
173 regression analysis.

174 Table 3 illustrates the findings of the multiple logistic regression analysis for the ASD-
175 associated factors. Parental education level, ethnicity, children's gender, and urinary Al
176 level were significant ASD risk factors. When compared to parents with secondary
177 education, parents with tertiary education had 17.9 times the odds of having ASD children
178 (adjusted odds ratio (aOR)=17.89 ; 95.0% confidence interval (CI): 5.97–53.63; p<0.001).
179 Compared to Malay children, non-Malay children had 6.1 times greater odds of having
180 ASD (aOR=6.11 ; 95.0% CI: 1.17 –31.96 ; p=0.032). Compared to girls, boys had 9.8
181 times greater odds of having ASD (aOR=9.82 ; 95.0% CI: 3.22 –30.00; p<0.001).
182 Compared to children with urinary Al levels of ≤1.6 µg/dL, children with urinary Al levels
183 of >1.6 µg/dL had 12.8 times greater odds of having ASD (aOR=12.77 ; 95.0% CI: 4.50
184 –36.27 ; p<0.001).

185 **Discussion**

186 The long-lasting and permanent human health effects of environmental neurotoxins have
187 become a great concern nowadays. It is due to the hazard characteristic of
188 neurotoxicants, which impair physiological function of organs at low concentrations and
189 cause permanent damage to the organ's morphological features. The presence of
190 neurotoxicants in environmental media (such as air, water, soil, food, and household

191   products), particularly in highly polluted areas such as urban cities and industrial zones,

192   would increase the population's risk of exposure. Young children, in particular, have more

193   potential to receive the effects of neurotoxicants than adults due to their immature organs'

194   ability to detoxify the hazardous chemicals in the body and develop important organs such

195   as the brain. The exposure to neurotoxins could increase due to their mouthing behavior

196   and curiosity (they tend to explore and put anything into their mouth), and food selectivity

197   (they are picky eaters, which leads to less intake of essential nutrition).

198   Children's being exposed to neurotoxicants, particularly Al at the critical developmental

199   stages (less than 6 years), may be related to the etiology of ASD. Different magnitudes

200   of exposure might affect the spectrum of the disease. We were able to demonstrate the

201   significant pattern of the autism spectrum in relation to urinary Al concentration. In this

202   study, there was a concrete finding between ASD severity and the accumulation level of

203   Al in the urine. Here, children with moderate-functioning ASD had the highest

204   concentration level of urinary Al, followed by that in children with high-functioning ASD

205   and TD children. This indicates higher Al exposure among children with more severe

206   ASD. To the current level of our knowledge, no study has ever demonstrated similar

207   findings pertaining to urinary Al and ASD. However, a study done in India revealed a

208   significant elevation of other neurotoxicants, lead (Pb) and mercury (Hg) in the hair and

209   nail samples as ASD becomes more severe (Priya & Geetha 2011).

210   Fundamentally, we were able to demonstrate that ASD children had greater urine Al

211   concentrations than TD children. This finding was supported by previous studies. For

212   instance, a study demonstrated significantly increased urinary Al levels in 55 Egyptian

213   ASD children (34.56 ± 20.88 µg/g creatinine) compared to 75 TD children matched by

214   age, gender, and socioeconomic status (17.56 ± 5.75 µg/g creatinine) (Metwally et al.

215   2015). Besides that, a study was done on 25 ASD children and 25 TD children matched

216   by age and gender in Egypt and reported that the ASD children had significantly higher

217   mean urinary Al (111.26 ± 142.94 µg/g creatinine) than the TD children (22.98 ± 21.29

218   µg/g creatinine) (Blaurock-Busch et al. 2011).

219   To date, no report has published a cut-off point for urinary Al level. It is important to
220   determine the safe level of Al for the reference in the human biomonitoring program. In
221   2004, the Florida Department of Health reported a wide range of urinary Al of 0.50 µg/dL
222   to 5.50 µg/dL among children in Suwanne County, Florida (ATSDR 2004). In this study,
223   we were able to determine the cut-off point of urinary Al of 1.60 µg/dL among pre-school
224   children in the urban city of Kuala Lumpur. It should be noted that the proposed cut-off
225   point was based on small sample size, and it has not been validated on an independent
226   dataset. However, this value may be useful for comparison in future studies conducted
227   among children living in other major cities in Malaysia, such as Johor Bahru (in Johor
228   state), Klang (in Selangor state), Georgetown (in Penang state), Kuching (in Sarawak
229   state), and Kota Kinabalu (in Sabah state).

230   The regression analysis in this study demonstrated urinary Al concentration as an
231   important risk factor for developing ASD among young children. Children with a urinary
232   Al concentration of >1.60 µg/dL had higher odds of developing ASD than children with a
233   urinary Al concentration of ≤1.60 µg/dL. In other words, higher urinary Al levels reflect
234   higher exposure to Al. A study done in Japan demonstrated higher urinary Al
235   concentration among Al-handling workers (median: 20.10 µg/g creatinine) than non-Al-
236   handling workers (median: 8.80 µg/g creatinine) (Ogawa & Kayama 2015). Another study
237   done in Sweden demonstrated the highest urinary Al concentrations among the exposed
238   workers (median 8.10 µmol/L) and lower concentrations among retired workers (median
239   0.80 µmol/L) and occupationally non-exposed workers (median 0.09 µmol/L) (Ljunggren
240   et al. 1991). The higher body burden of Al eventually impaired the function of the brain
241   and could be linked to neurobehavioral diseases, particularly ASD (Mold et al. 2018;
242   Morris et al. 2017).

243   The findings from this study also indicate that the assessment of Al in relation to ASD can
244   be done through urine sample analysis and the finding is reliable. Besides that, urine
245   collection is preferable among young children over blood sampling due to its non-invasive
246   procedure. Assessment of Al in the urine may indicate acute exposure (Xu et al. 1991).
247   However, it may also recur over a long period as a result of frequent exposure through

248   oral ingestion and inhalation. Nonetheless, elimination of Al may require years and could

249   be due to Al retention in body storage from which it is removed progressively (Priest et al.

250   1995). Most likely, this storage occurs in the bone, which contains approximately 60.0%

251   of the human Al body burden (Krewski et al. 2007). It is also predicted that slow Al removal

252   accompanied by continued exposure would produce an increasing body burden with age

253   (Yokel & McNamara 2001). In other words, a long-term exposure to Al gives rise to an

254   accumulation of Al in the body and skeleton of healthy persons, and that the elimination

255   of retained Al is very slow, in the order of several years (Elinder et al. 1991; Ljunggren et

256   al. 1991). **The results of this study and the evidence listed above** can be used as

257   reference and supporting evidence upon the proposal and development of human

258   biomonitoring program in Malaysia.

259   The main strategies for reducing exposure to Al should include the environmental health

260   approach. The government should strengthen preventive measures for controlling and

261   monitoring Al levels in food and water. For instance, currently, heavy metal contaminants

262   in food products are monitored by the Food Safety and Quality Division, MOH Malaysia,

263   while the water quality is controlled and monitored by the water supply concessionaire

264   and the Drinking Water Quality Control Unit, MOH Malaysia. Instant action should be

265   taken when Al concentrations in food and drinking water exceed safe levels, for example,

266   by withdrawing the product from the market and halting the supply of public drinking water

267   until remedial action is taken.

268   While environmental monitoring of pollutants in specific environmental media (air, water,

269   soil, food, and domestic products) is important for the control and prevention of exposure,

270   it cannot reflect the real exposure received by humans. Many developed countries have

271   implemented human biomonitoring to monitor human exposure to environmental

272   pollutants (WHO 2015). Human biomonitoring allows us to measure exposure to

273   pollutants by measuring either the substances themselves, their metabolites, or markers

274   of subsequence health effects in body fluid or tissues. At present, there is no established

275   human biomonitoring program to monitor the exposure of environmental toxicants among

276   the Malaysian population. Therefore, the MOH Malaysia should introduce the first ever

277 human biomonitoring program in Malaysia in order to monitor the body burden of
278 environmental toxicants including Al among the population. The program could be
279 initiated in high-risk areas such as urban cities and industrialized zones. In terms of
280 sensitive receptors, vulnerable groups such as children, pregnant women, and the elderly
281 should be considered and included in the monitoring program. The monitoring of
282 environmental toxicants can be done through urine samples compared to blood/plasma
283 samples among children in view of the non-invasive procedure, easy to collect, non-
284 expensive, and requiring fewer human resources. The determination of Al in urine is
285 preferred for conducting biological monitoring due to its better sensitivity and robustness
286 when compared to the detection of Al in plasma (Ogawa & Kayama 2015). To run this
287 program, MOH Malaysia should be ready in term of financial support, laboratory capacity
288 and human resources.

289 There are limitations to this study, and the results should be interpreted with care. Only
290 Al was tested in urine, which may not fully explain the complicated pathogenic pathways
291 in the brain caused by this neurotoxicant. Moreover, the authors did not include other
292 neurotoxicants like organophosphate pesticides, heavy metals (such as mercury (Hg),
293 lead (Pb), cadmium (Cd), arsenic (As), manganese (Mn), and chromium (Cr)), and air
294 pollutants (such as volatile organic compounds (VOCs), polyaromatic hydrocarbons
295 (PAHs), particulate matter $(PM)_{10}$ and $PM_{2.5}$, and nitrogen oxides (NO_x)). These
296 neurotoxic elements undoubtedly affect neurodevelopment in children. Nonetheless, the
297 results found in this study indicate that further studies are necessary to examine the
298 possible action of Al and other neurotoxicants in ASD. Other than that, the ASD and TD
299 groups lacked comparability for ethnicity, children's gender, and socioeconomic status.
300 This imbalanced distribution between each group of children might cause a biased
301 approximation of the effect of urinary Al on ASD. However, the regression analysis was
302 performed to overcome this issue. Lastly, ASD children recruited in this study were
303 comprised of high-functioning ASD and moderate-functioning ASD but not low-
304 functioning ASD due to the unavailability of this group in *Genius Kurnia* center, as this
305 institution only recruited children with high-functioning ASD and moderate-functioning

3333333

306 ASD for early intervention. If children with low-functioning ASD were included in the study,

307 there would be more valuable findings discovered.

Conclusions

309 Higher urinary Al levels over 1.60 µg/dL were discovered to be a significant risk factor for

310 ASD in preschool children in Kuala Lumpur. It suggests that increased exposure to Al

311 may occur, and that ASD may be associated with the availability and neurotoxic effect of

312 Al in the brain. Future studies are needed to explore the relationships of other clinical

313 samples of Al, such as blood, nails, and hair, with ASD among preschool children in

314 different urban cities in Malaysia.

Acknowledgements

316 The authors thank the UKM Department of Community Health and the Faculty of Medicine

317 for their assistance in this research. The authors would also like to thank *Genius Kurnia*,

318 Ministry of Education Malaysia, and *Kemas*, Ministry of Rural Development Malaysia, for

319 allowing permission to perform this research.

320

References

322 Abd Wahil MS, Ja'afar MH, and Isa ZM. 2021. Assessment of Urinary Lead (Pb) and

323   Essential Trace Elements in Autism Spectrum Disorder: a Case-Control Study

324   Among Preschool Children in Malaysia. *Biological trace element research*:1-25.

325   DOI 10.1007/s12011-021-02654-w.

326 Al-Ayadhi LY, Ben Bacha AG, Kotb M, and El-Ansary AK. 2012. A novel study on amyloid

327   β peptide 40, 42 and 40/42 ratio in Saudi autistics. *Behavioral and Brain Functions*

328   8:1-8. DOI 10.1186/1744-9081-8-4.

329   APA. 2013. *Diagnostic and statistical manual of mental disorders (DSM-5®)*: American
330   Psychiatric Pub.

331   ATSDR. 2004. Blood and Urine Tests. Live Oak, Suwannee Country, Florida.
332   https://www.atsdr.cdc.gov/hac/pha/bloodurinetestsresults051104-
333   fl/bloodurinetestsresultshc051104.pdf. Accessed 18 October 2021.

334   ATSDR.   2008.   Toxicological   profile   for   aluminum.
335   https://www.atsdr.cdc.gov/toxprofiles/tp22.pdf. Accessed 22 October 2021.

336   Banks WA, Niehoff ML, Drago D, and Zatta P. 2006. Aluminum complexing enhances
337   amyloid β protein penetration of blood–brain barrier. *Brain research* 1116:215-221.
338   DOI 10.1016/j.brainres.2006.07.112.

339   Baron-Cohen S, Scott FJ, Allison C, Williams J, Bolton P, Matthews FE, and Brayne C.
340   2009. Prevalence of autism-spectrum conditions: UK school-based population
341   study. *The   British   Journal   of   Psychiatry*   194:500-509.   DOI
342   10.1192/bjp.bp.108.059345.

343   Bearer CF. 1995. Environmental health hazards: how children are different from adults.
344   *The Future of Children*:11-26. DOI 10.2307/1602354.

345   Becaria A, Bondy SC, and Campbell A. 2003. Aluminum and copper interact in the
346   promotion of oxidative but not inflammatory events: implications for Alzheimer's
347   disease. *Journal of Alzheimer's Disease* 5:31-38. DOI 10.3233/jad-2003-5105.

348   Blaurock-Busch E, Amin OR, and Rabah T. 2011. Heavy metals and trace elements in
349   hair and urine of a sample of arab children with autistic spectrum disorder. *Maedica*
350   6:247. PMID 22879836.

351   Blumberg SJ, Bramlett MD, Kogan MD, Schieve LA, Jones JR, and Lu MC. 2013.
352   *Changes in prevalence of parent-reported autism spectrum disorder in school-
353   aged US children: 2007 to 2011-2012*: US Department of Health and Human
354   Services, Centers for Disease Control and Prevention, National Center for Health
355   Statistic. PMID 24988818.

356 Bölte S, Girdler S, and Marschik PB. 2019. The contribution of environmental exposure
357   to the etiology of autism spectrum disorder. *Cellular and Molecular Life Sciences*
358   76:1275-1297. DOI 10.1007/s00018-018-2988-4.

359 Bondy SC. 2016. Low levels of aluminum can lead to behavioral and morphological
360   changes associated with Alzheimer's disease and age-related neurodegeneration.
361   *Neurotoxicology* 52:222-229. DOI 10.1016/j.neuro.2015.12.002.

362 Bondy SC, and Campbell A. 2017. Aluminum and neurodegenerative diseases.
363   *Advances in Neurotoxicology*: Elsevier, 131-156. DOI
364   10.1016/bs.ant.2017.07.008.

365 Campbell A. 2002. The potential role of aluminium in Alzheimer's disease. *Nephrology*
366   *Dialysis Transplantation* 17:17-20. DOI 10.1093/ndt/17.suppl_2.17.

367 Chugh S, Arora V, Kaur S, and Sood A. 1993. Toxicity of exposed aluminium phosphide.
368   *The Journal of the Association of Physicians of India* 41:569-570. PMID 8307912.

369 Corkins MR. 2019. Aluminum effects in infants and children. *Pediatrics* 144. DOI
370   10.1542/peds.2019-3148.

371 Crisponi G, Fanni D, Gerosa C, Nemolato S, Nurchi VM, Crespo-Alonso M, Lachowicz JI,
372   and Faa G. 2013. The meaning of aluminium exposure on human health and
373   aluminium-related diseases. *Biomolecular concepts* 4:77-87. DOI 10.1515/bmc-
374   2012-0045.

375 Di J, and Bi S. 2004. Aluminum facilitation of the iron-mediated oxidation of DOPA to
376   melanin. *Analytical sciences* 20:629-634. DOI 10.2116/analsci.20.629.

377 Elinder C-G, Ahrengart L, Lidums V, Pettersson E, and Sjögren B. 1991. Evidence of
378   aluminium accumulation in aluminium welders. *Occupational and environmental*
379   *medicine* 48:735-738. DOI 10.1136/oem.48.11.735.

380 Exley C. 2009. Darwin, natural selection and the biological essentiality of aluminium and
381   silicon. *Trends in Biochemical Sciences* 34:589-593. DOI
382   10.1016/j.tibs.2009.07.006.

383   Ferretti G, Marchionni C, Bacchetti T, Galeazzi T, and Dousset N. 2003. Effect of
384   aluminium on lipid peroxidation of human high density lipoproteins. *Free radical*
385   *research* 37:515-521. DOI 10.1080/1071576031000083125.

386   Fombonne E. 2003. The prevalence of autism. *Jama* 289:87-89. DOI
387   10.1001/jama.289.1.87.

388   Freedman S. 2010. *Developing college skills in students with autism and Asperger's*
389   *syndrome*: Jessica Kingsley Publishers. ISBN 9781843109174.

390   Gale CR, O'Callaghan FJ, Godfrey KM, Law CM, and Martyn CN. 2004. Critical periods
391   of brain growth and cognitive function in children. *Brain* 127:321-329. DOI
392   10.1093/brain/awh034.

393   Kanner L. 1943. Autistic disturbances of affective contact. *Nervous child* 2:217-250.

394   Kaur J, Engkasan J, Sivanesom R, Bahar N, Noorand M, and Kamarudin K. 2015.
395   Technical report autism spectrum disorder research in Malaysia. *Malaysia: Ministry*
396   *of Health Malaysia*. ISBN 978-983-2387-10-7.

397   Krewski D, Yokel RA, Nieboer E, Borchelt D, Cohen J, Harry J, Kacew S, Lindsay J,
398   Mahfouz AM, and Rondeau V. 2007. Human health risk assessment for aluminium,
399   aluminium oxide, and aluminium hydroxide. *Journal of Toxicology and*
400   *Environmental Health, Part B* 10:1-269. DOI 10.1080/10937400701597766.

401   Kumar V, and Gill KD. 2014. Oxidative stress and mitochondrial dysfunction in aluminium
402   neurotoxicity and its amelioration: a review. *Neurotoxicology* 41:154-166. DOI
403   10.1016/j.neuro.2014.02.004.

404   Ljunggren KG, Lidums V, and Sjögren B. 1991. Blood and urine concentrations of
405   aluminium among workers exposed to aluminium flake powders. *Occupational and*
406   *environmental medicine* 48:106-109. DOI 10.1136/oem.48.2.106.

407   Metwally FM, Abdelraoof ER, Hasheesh A, Elsedfy ZB, Gebril O, Meguid N, and Rashad
408   H. 2015. Toxic effect of some heavy metals in Egyptian autistic children.
409   *International Journal of Pharmaceutical and Clinical Research* 7:206-211.

410 Mold M, Umar D, King A, and Exley C. 2018. Aluminium in brain tissue in autism. *Journal*
411 *of Trace Elements in Medicine and Biology* 46:76-82. DOI
412 10.1016/j.jtemb.2017.11.012

413 Morris G, Puri BK, and Frye RE. 2017. The putative role of environmental aluminium in
414 the development of chronic neuropathology in adults and children. How strong is
415 the evidence and what could be the mechanisms involved? *Metabolic brain*
416 *disease* 32:1335-1355. DOI 10.1007/s11011-017-0077-2.

417 Ogawa M, and Kayama F. 2015. A study of the association between urinary aluminum
418 concentration and pre-clinical findings among aluminum-handling and non-
419 handling workers. *Journal of Occupational Medicine and Toxicology* 10:13. DOI
420 10.1186/s12995-015-0055-8.

421 Priest N, Newton D, Day J, Talbot R, and Warner A. 1995. Human metabolism of
422 aluminium-26 and gallium-67 injected as citrates. *Human & experimental*
423 *toxicology* 14:287-293. DOI 10.1177/096032719501400309.

424 Priest ND. 1993. The bioavailability and metabolism of aluminium compounds in man.
425 *Proceedings of the Nutrition Society* 52:231-240. DOI 10.1079/pns19930055.

426 Priya MDL, and Geetha A. 2011. Level of trace elements (copper, zinc, magnesium and
427 selenium) and toxic elements (lead and mercury) in the hair and nail of children
428 with autism. *Biological trace element research* 142:148-158. DOI 10.1007/s12011-
429 010-8766-2

430 Rahbar MH, Samms-Vaughan M, Dickerson AS, Loveland KA, Ardjomand-Hessabi M,
431 Bressler J, Shakespeare-Pellington S, Grove ML, Pearson DA, and Boerwinkle E.
432 2015. Blood lead concentrations in Jamaican children with and without autism
433 spectrum disorder. *International journal of environmental research and public*
434 *health* 12:83-105. DOI 10.3390/ijerph120100083

435 Rice D, and Barone Jr S. 2000. Critical periods of vulnerability for the developing nervous
436 system: evidence from humans and animal models. *Environmental health*
437 *perspectives* 108:511-533. DOI 10.1289/ehp.00108s3511.

438 Sánchez-Iglesias S, Méndez-Álvarez E, Iglesias-González J, Muñoz-Patiño A,
439   Sánchez-Sellero I, Labandeira-García JL, and Soto-Otero R. 2009. Brain oxidative
440   stress and selective behaviour of aluminium in specific areas of rat brain: potential
441   effects in a 6-OHDA-induced model of Parkinson's disease. *Journal of*
442   *neurochemistry* 109:879-888. DOI 10.1111/j.1471-4159.2009.06019.x.

443 Verstraeten SV, Aimo L, and Oteiza PI. 2008. Aluminium and lead: molecular
444   mechanisms of brain toxicity. *Archives of toxicology* 82:789-802. DOI
445   10.1007/s00204-008-0345-3.

446 WHO. 2004. ICD-10 : International Statistical Classification of Diseases and Related
447   Health Problems : Tenth Revision. 2nd ed ed. Geneva: World Health Organization.

448 WHO. 2006. Principles for Evaluating Health Risks in Children Associated with Exposure
449   to   Chemicals.   Environmental   Health   Criteria   237.
450   https://inchem.org/documents/ehc/ehc/ehc237.pdf. Accessed 17 November 2021.

451 WHO. 2015. Regional Office for Europe. Human biomonitoring: facts and figures.
452   Copenhagen.http://www.euro.who.int/__data/assets/pdf_file/0020/276311/Huma
453   n-biomonitoring-facts-figures-en.pdf. Xu ZX, Pai SM, and Melethil S. 1991.
454   Kinetics of aluminum in rats. II: Dose-dependent urinary and biliary excretion.
455   *Journal of pharmaceutical sciences* 80:946-951. DOI 10.1002/jps.2600801009.

456 Yokel RA, and McNamara PJ. 2001. Aluminium toxicokinetics: an updated minireview.
457   *Pharmacology & Toxicology: MiniReview* 88:159-167. DOI 10.1034/j.1600-
458   0773.2001.d01-98.x.

459 Yokel RA, Rhineheimer SS, Sharma P, Elmore D, and McNamara PJ. 2001. Entry, half-
460   life, and desferrioxamine-accelerated clearance of brain aluminum after a single
461   [26]Al exposure. *Toxicological sciences* 64:77-82. DOI 10.1093/toxsci/64.1.77.

462 Yokel RA, Wilson M, Harris WR, and Halestrap AP. 2002. Aluminum citrate uptake by
463   immortalized brain endothelial cells: implications for its blood–brain barrier
464   transport. *Brain research* 930:101-110. DOI 10.1016/s0006-8993(02)02234-5.

465

466
467

Respondents' characteristics for both case and control groups.

1   Table 1. Respondents' characteristics for both case and control groups.

	ASD (n = 81)	TD (n = 74)		
Variables	n (%)	n (%)	x^2-value	p-value
Parental Background				
Education Level				
Secondary Education	12 (14.8)	45 (60.8)	35.187	**<0.001***
Tertiary Education	69 (85.2)	29 (39.2)		
Mean (± SD) Monthly Income in Ringgit Malaysia (RM)	6699.94 ± 3856.52	3960.03 ± 2025.19	-5.461#	**<0.001***
Median (IQR) Monthly Income in (RM)	5500.00 (6500.00)	3500.00 (2575.00)	-4.867ε	**<0.001***
Income Classification				
≤ RM5000.00	39 (48.1)	58 (78.4)	17.922	**<0.001***
> RM5000.00	42 (51.9)	16 (21.6)		
Children Background				
Ethnicity				
Malay	63 (77.8)	70 (94.6)	8.980	**0.003***
Non-Malay	18 (22.2)	4 (5.4)		
Gender				
Male	68 (84.0)	39 (52.7)	17.663	**<0.001***
Female	13 (16.0)	35 (47.3)		
Mean (± SD) Age (Years)	5.63 ± 0.60	5.45 ± 0.83	-1.188#	0.114
Median (IQR) Age (Years)	6.00 (1.00)	6.00 (1.00)	-1.163ε	0.245
Age Group				
≤ 4 years old	5 (6.2)	10 (13.5)	2.384	0.123
> 4 years old	76 (93.8)	64 (86.5)		

2   * p < 0.05 indicates significant statistical result

3   # t-value from Student's T-Test to determine comparison of mean (SD) of the continuous variables

4   between ASD and TD group

5   ε z-value from Mann-Whitney U Test to determine comparison of median (IQR) of the continuous variables

6   between ASD and TD group

Comparison of the urinary Al concentration between case and control groups

1   Table 2. Comparison of the urinary Al concentration between case and control groups

Urinary Al level	ASD	TD	p-value#
No. of Respondents (%)	81 (52.26 %)	74 (47.74 %)	
Minimum (µg/dL)	0.04	0.001	
Maximum (µg/dL)	47.24	24.10	
Mean (± SD) (µg/dL)	5.06 (± 6.30)	2.58 (± 4.89)	
Geometric Mean (µg/dL)	2.93	0.27	
Median (IQR) (µg/dL)	2.89 (6.77)	0.96 (2.95)	**< 0.001*#**

2   * p < 0.05 indicates significant statistical result

3   # Comparison of median (IQR) urinary Al concentration between ASD and TD groups using Mann Whitney

4   U Test

Multiple logistic regression analysis of associated factors for ASD

1   Table 3. Multiple logistic regression analysis of associated factors for ASD

Variables	B	Wald	aOR	95.0 % CI	p-value
Parental Background					
Education Background					
Tertiary Education	2.88	26.52	17.89	5.97, 53.63	**<0.001***
Secondary Education			1.00		
Children Background					
Ethnicity					
Non-Malay	1.81	4.60	6.11	1.17, 31.96	**0.032***
Malay			1.00		
Gender					
Male	2.29	16.08	9.82	3.22, 30.00	**<0.001***
Female			1.00		
Heavy Metal					
Urinary Al					
> 1.6 µg/dL	2.55	22.88	12.77	4.50, 36.27	**<0.001***
≤ 1.6 µg/dL			1.00		

2   * $p < 0.05$ indicates significant statistical result from multiple logistic regression using stepwise method

3   aOR = adjusted odds ratio; CI = confidence interval

Figure 1

Selection of the respondents into the study
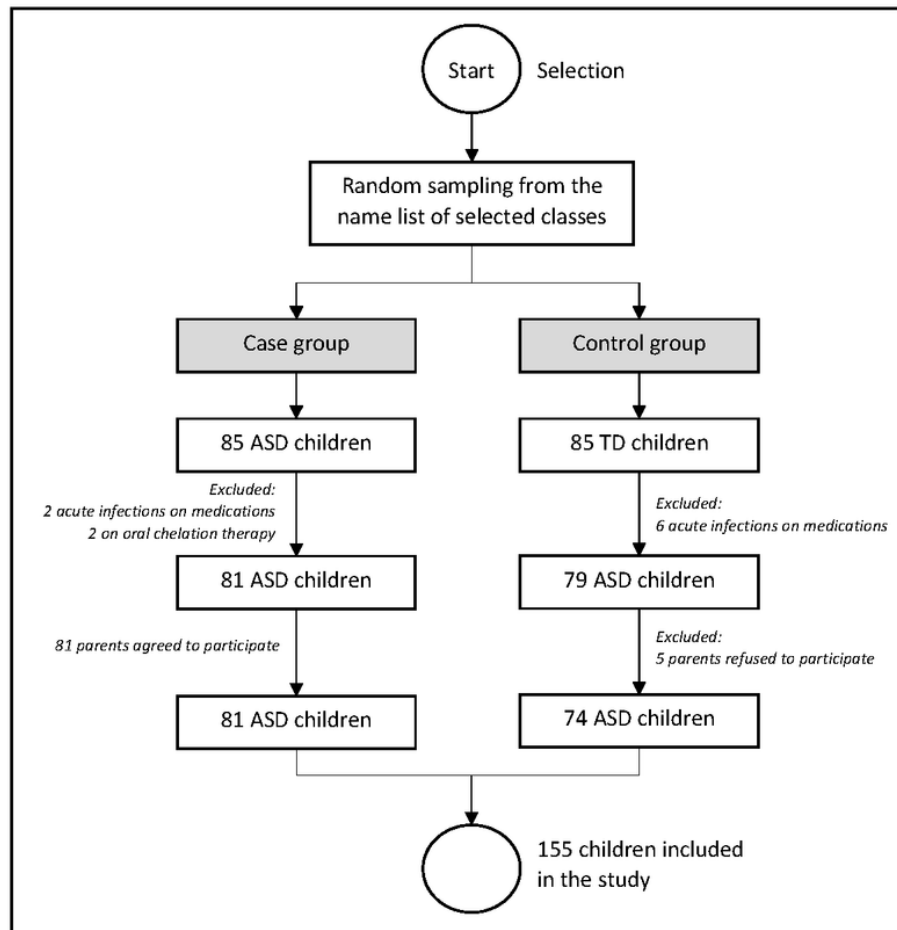
Figure 2

Comparison of the median urinary Al concentration according to the severity of ASD.

* Comparison of median urinary Al concentration between degree of disorder severity using Kruskal Wallis test
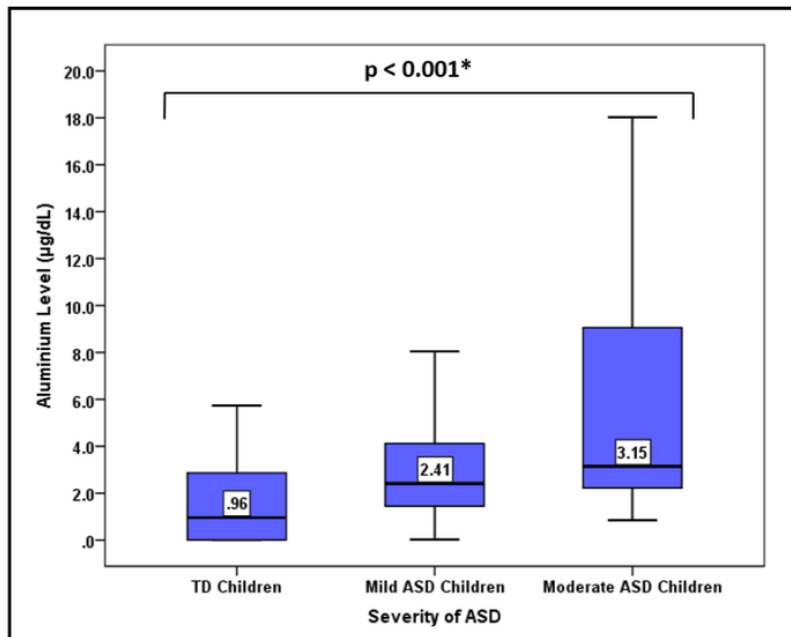
Figure 3

Receiver Operating Characteristic (ROC) Curve Analysis of urinary Al and ASD.