



Title	Delta-aminolevulinic acid dehydratase (ALAD) and vitamin D receptor (VDR) genes polymorphisms in children residing in an abandoned lead-zinc mine area in Kabwe, Zambia
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1 Delta-aminolevulinic acid dehydratase (ALAD) and vitamin D receptor (VDR) genes
2 polymorphisms in children residing in an abandoned lead-zinc mine area

3
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29 **Abstract**

30 Lead is a ubiquitous environmental pollutant that poses serious health problems to humans,
31 especially to children. However, genetic variability in individuals varies their susceptibility to lead
32 poisoning. One possible factor is genetic polymorphism. Thus, this study aimed to investigate the
33 association between blood lead level (BLL), and polymorphisms in the delta-aminolevulinic acid
34 dehydratase (ALAD) *MspI* (rs1800435) and vitamin D receptor (VDR) *FokI* (rs19735810), *BsmI*
35 (rs15444410), *ApaI* (rs7975232) and *TaqI* (rs731236) genes in children exposed to lead. A total of
36 140 children (aged 2 - 10 years) were recruited in areas living closer to and far away from an
37 abandoned lead-zinc mine in Kabwe, Zambia. Blood samples were collected from each child for
38 BLLs and polymorphisms analysis.

39 All children were homozygous for the ALAD 1 allele, indicating there might be bioavailable lead in
40 the children's blood which can transfer to the soft tissues and the brain. The distribution of the VDR
41 gene polymorphisms showed major alleles prevalence's of 81%, 80%, 68%, and 75% for *FokI*, *BsmI*,
42 *ApaI*, and *TaqI* polymorphisms, respectively. The aa genotype of VDR *ApaI* showed significantly
43 higher BLL compared to other genotypes of the VDRs polymorphism. The *TaqI* - TT genotype was
44 associated with an increase of lead exposure risk in female children (OR = 2.06; 95% CI:1.04 – 4.06,
45 $p = 0.03$). The haplotype analysis showed 10 haplotypes with a frequency above 1%, and the *FbAt*
46 haplotype showed a protective role against lead toxicity. In conclusion, the children, especially
47 female children, which exposed to lead mainly from the abandoned lead-zinc mine might be at a
48 higher risk of developing lead poisoning. Further, larger scale sample sizes are needed to corroborate
49 the role of ALAD and VDR genetic variants on the implications of lead toxicity in the general
50 population, particularly in children.

51 **Keywords:** Lead; Gene polymorphism; ALAD; VDR; Children

52 **1. Introduction**

53 Lead is a ubiquitous environmental contaminant that caused adverse health problems and widespread
54 environmental contamination. To date, no safe blood lead-exposure threshold has been identified.
55 Children compared to adults are particularly susceptible, and at a greater risk to the neurotoxic
56 effects of lead (Sanders et al., 2010). Lead can cause damage to children's cognitive functioning that
57 persist into adulthood and in some cases irreversible neurological damage (Flora et al., 2012;
58 Lanphear et al., 2005;). There is an inter-individual variation in susceptibility to the adverse health
59 effects of lead and molecular evidence has indicated that genetic factors such as gene polymorphism
60 can modify lead toxicity or might be protective from lead poisoning (Kim et al., 2014). The delta-
61 aminolevulinic acid dehydratase (δ -ALAD) and vitamin D receptor (VDR) have shown potential as
62 candidate genes for lead susceptibility in humans (Onalaja and Claudio, 2000).

63 Aminolevulinic acid dehydratase, the second enzyme in the heme biosynthesis, is a polymorphic
64 enzyme with two codominant alleles called ALAD 1 and ALAD 2 (Wetmur et al., 1991a). The
65 difference between these two polypeptides is a single nucleotide polymorphism (SNP) results in the
66 substitution of neutral asparagine for positively charged lysine at position 177 of the coding region
67 (rs1800435). The biological explanation to this substitution makes the ALAD 2 enzyme more
68 electronegative than the ALAD 1 and may have a greater affinity for lead ions (Battistuzzi et al.,
69 1981; Wetmur et al., 1991a). Several studies reported higher blood lead concentrations in the ALAD
70 2 allele carriers than the ALAD 1 allele carriers (Montenegro et al., 2006; Wetmur et al., 1991b),
71 suggesting the individual variation in susceptibility to lead exposure.

72 Lead can substitute calcium in many biological systems since both are divalent cations. Thus, lead
73 can exert toxic effects by mimicking the actions of calcium and interact with proteins (Tchounwou
74 et al., 2012). The high binding capacity of lead to calcium-binding proteins suggested that at low

75 calcium intake lead absorption can increase in our body. [Bruening et al. \(1999\)](#) reported significantly
76 lower dietary calcium intake in children with elevated blood lead levels. The vitamin D endocrine
77 system and the VDR are vital parts for calcium homeostasis. And in humans, several VDR SNPs
78 have been identified ([Uitterlinden et al., 2004a](#)). Among these several SNPs, the *Apal* - rs7975232,
79 *BsmI* - rs1544410, *FokI* - rs2228570, and *TaqI* - rs731236 were investigated intensively for their
80 association with altered calcium metabolism and human traits ([Uitterlinden et al., 2004b](#), [Valdivielso](#)
81 [and Fernandez, 2006](#)).

82 Lead exposure represents a significant contributor to adverse health effects in developing countries.
83 Children's deaths due to lead poisoning were reported from the recycling of used lead-acid battery
84 in Senegal ([Haefliger et al., 2008](#)) and artisanal gold mining in Nigeria ([Dooyema et al., 2011](#)).
85 Because of the abandoned lead-zinc mine in the town which operated for almost a century, Kabwe -
86 Zambia present in a list of the top ten world's most polluted places ([Blacksmith Institute, 2013](#)).
87 Yabe et al. ([2015](#)) has reported blood lead levels up to 427.8 µg/dL in children residing close to the
88 closed mine in Kabwe. Besides, previous studies reported very high lead concentrations in different
89 biological and environmental matrices ([Nakayama et al., 2011](#); [Toyomaki et al., 2020](#); [Yabe et al.,](#)
90 [2011, 2013, 2020](#);). However, regardless of the high burden of lead exposure in Kabwe town, gaps
91 still exist in the knowledge of lead exposure and its impact on children. To our knowledge, there is
92 only one study that investigated the methylation status of ALAD and p16 genes in children exposed
93 to environmental lead from Kabwe, Zambia ([Yohannes et al., 2020](#)). Nevertheless, no data exist
94 concerning the impacts of lead on genetic variants among the people from Kabwe.

95 In this study, we aimed to investigate the associations of ALAD *MspI* (rs1800435), VDR – *Apal*
96 (rs7975232), *BsmI* (rs1544410), *FokI* (rs2228570), and *TaqI* (rs731236) polymorphisms with blood
97 lead concentration in children living closer to or/and distance away from an abandoned lead-zinc

98 mine in Kabwe, Zambia. As there is no information about gene polymorphism in association with
99 the lead exposure in Kabwe, the present study is one of the first efforts and the result of this study
100 will serve as baseline information for future researches.

101

102

103 **2. Materials and Methods**

104 *2.1. Study subjects and sampling*

105 The sampling area information and demographic data of the recruited children have been published
106 in our previous study ([Yohannes et al., 2020](#)). Participation in this study was voluntary. The study
107 recruited a total of 140 randomly chosen children from five townships called Bwacha, Chowa,
108 Kasanda, Makululu, and Nakoli in Kabwe town, Zambia. The children's parents or guardians signed
109 written informed consent forms before the enrollment.

110

111 The study was conducted in July 2016. From each child, 3 mL of the venous blood sample was
112 collected into vacuum tubes containing heparin. The BLLs were measured using a portable LeadCare
113 II analyzer after blood collection that same day as described by [Yohannes et al. \(2020\)](#). For genetic
114 analysis, the blood samples were immediately stored at $-20\text{ }^{\circ}\text{C}$ after lead analysis. After obtaining a
115 material transfer agreement (MTA, Approval No. E00417) from the Ministry of Health, Zambia, the
116 frozen samples were transported to Japan. All the genetic analyses were carried out in the Laboratory
117 of Toxicology, Faculty of Veterinary Medicine, Hokkaido University, Japan.

118

119 2.2. Genetic analyses

120 Genomic DNA was isolated from whole blood using the NucleoSpin Blood Kit (Macherey-Nagel,
121 Duren, Germany) according to the manufacturer's instructions. The concentration and quality of
122 genomic DNA were assessed using a NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Fisher
123 Scientific Inc., USA). The purified DNA was kept at -20 °C until genotyping was conducted.

124 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed
125 for the genotyping of ALAD - *MspI* (G/C), VDR - *BsmI* (G/A), *FokI* (T/C), *ApaI* (G/T), and *TaqI*
126 (T/C). The ALAD polymorphism (917 bp) was performed using primers: 5'-
127 AGACAGACATTAGCTCAGTAGAGG-3' and 5'-GGCAAAGACCACGTCCATTAC-3'.
128 Genomics for the *BsmI* polymorphism (831 bp) was determined using primers: 5'-
129 GACCTGTGGCAACCAAGACT-3' and 5'-AACCAGCGGAAGAGGTCAAG-3'; *FokI*
130 polymorphism (267 bp) using primers: 5'-AGCTGGCCCTGGCACTGACTCTGGCT-3' and 5'-
131 ATGGAAACACCTTGCTTCTTCTCCCTC-3'; and *ApaI* and *TaqI* polymorphisms (832 bp) using
132 primers: 5'-CAGAGCATGGACAGGGAGCAA-3' and 5'-AGGCAGCGGTGGAGGCATCTCT-3'.
133 PCR reaction was performed in a 20 µL reaction mixture containing 0.3 µmol/L of each primer, 500
134 ng of genomic DNA and 10 µL EmeraldAmp PCR Master Mix (2x premix, TaKaRa Bio, Japan).
135 PCR conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of
136 denaturation at 94 °C for 30 s, annealing at 62 °C for ALAD and *BsmI*, and 68 °C for *FokI*, *ApaI*
137 and *TaqI* for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. The
138 DNA bands were visualized under a Blue-LED transilluminator system and photographed.

139

140

141 The resulting amplified products were subjected to restriction digestion using their respective
142 enzymes obtained from New England Biolabs (NEB). The samples were incubated for 4 h at 65 °C
143 for *BsmI* and *TaqI*, and heat inhibited for 20 min at 80 °C; incubated for 4 h at 37 °C for *ApaI* and
144 *FokI*, and heat inhibited for 20 min at 65 °C; incubated for 4 h at 37 °C for *MspI* (ALAD) with no
145 heat inactivation step. Digestion of the ALAD amplified product by *MspI* produces fragments of 584
146 bp and 158 bp for the ALAD 1-1, 584 bp, 513 bp and 158 bp for ALAD 1-2, and 513 and 158 bp for
147 the ALAD 2-2 genotypes. The VDR Alleles were designated by capital letters A, B, F, and T in the
148 absence of restriction site and by small letters a, b, f, and t for the presence of restriction site for
149 *ApaI*, *BsmI*, *FokI*, and *TaqI* polymorphisms, respectively. The genotypes and fragment lengths were
150 832 bp for AA, 832 bp, 615 bp and 217 bp for Aa, and 615 bp and 217 bp for aa genotypes; 831 bp
151 for BB, 831 bp, 655 bp and 176 bp for Bb, and 655 bp and 176 bp for bb genotypes; 267 bp for FF,
152 267 bp, 197 bp and 70 bp for Ff, and 197 bp and 70 bp for ff genotypes; 494 bp and 338 bp for TT,
153 498 bp, 338 bp, 293 bp and 201 bp for tt, and 338 bp, 293 bp and 201 bp for tt genotypes. The details
154 of nucleotide sequence, primer sets, and genotypes with fragment sizes after RFLP are presented as
155 **Supplementary Material**. Digested products were visualized after agarose gel electrophoresis
156 under a Blue-LED transilluminator system. Gel electrophoresis showed the identification of
157 genotypes of each VDR SNPs has been presented in Figure 1.

158

159

160 **Fig. 1.**

161

162

163 2.3. Statistical analysis

164 The JMP Pro 14 (SAS Institute, Cary, NC, USA) software was used for statistical analysis, and a p -
165 value < 0.05 was considered significant. The distribution of genotypes and allele frequencies among
166 the studied children was analyzed for Hardy-Weinberg equilibrium (HWE) by the chi-square test
167 (χ^2), and the genotype and allele frequencies were compared in relative to BLL quartiles. The
168 comparison of allele frequencies between the studied subjects and other studies with different
169 populations was performed by pairwise chi-square test. The odds ratios (ORs) with 95% confidence
170 interval (CI) were calculated to compare the risk of lead toxicity in low and high BLL level based
171 on the first quartile BLL, and between females and males. Due to low frequencies of aa, BB, ff, and
172 tt genotypes, we grouped *Apal* genotype as AA and (Aa + aa) groups; *BsmI* genotype as (BB + Bb)
173 and bb groups; *FokI* genotype as FF and (Ff + ff) groups, and *TaqI* genotype as TT and (Tt + tt)
174 groups. SNPStats software (available online at <http://bioinfo.iconcologia.net/SNPstats>).was used to
175 perform haplotype analysis and analyze the association with BLL.

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184 **3. Results**

185 A total of 140 children, 75 males and 65 females aged between 2 and 10 years, were engaged in the
186 present study (Yohannes et al., 2020). The mean ages of children were 5.7 ± 1.78 years for females
187 and 5.6 ± 1.88 years for males. The average BLL was 19.4 ± 10.6 $\mu\text{g/dL}$ and ranged from 1.65 to
188 60.8 $\mu\text{g/dL}$ among the children. There was no significant difference in BLL between sexes ($p =$
189 0.236) with mean values of 20.5 ± 12.4 $\mu\text{g/dL}$ and 18.4 ± 8.82 $\mu\text{g/dL}$ in female and male children,
190 respectively.

191

192 **3.1. Prevalence of ALAD and VDR SNPs, and haplotype frequencies of VDR genes**

193 The alleles and genotypes frequencies (%) of ALAD and VDR genes SNPs among the studied
194 children from Kabwe, Zambia are summarized in Table 1. All loci follow the Hardy-Weinberg
195 equilibrium ($p > 0.05$). Molecular analysis revealed that all the children tested for the ALAD
196 genotype were 100% ALAD 1-1. The overall prevalence of the VDR SNPs allele frequencies of A/a,
197 B/b, F/f, and T/t for the *ApaI*, *BsmI*, *FokI*, and *TaqI* polymorphisms were 0.68/0.32, 0.20/0.80,
198 0.81/0.19, and 0.75/0.25, respectively. For VDR-*ApaI*, a significantly higher percentage of allele a
199 (39%) was observed at the Q4 group with BLL > 25 $\mu\text{g/dL}$ (Table 1).

200

201

202 **Table 1**

203

204 3.2.Evaluation of VDR SNPs genotypes distribution between this study children and other studied
205 populations

206 The distribution of the four VDR SNPs data was available from other studies and the International
207 HapMap project populations (<http://hapmap.ncbi.nlm.nih.gov/>). A pairwise chi-square test was
208 conducted to compare the alleles frequencies of the present study with different populations. The
209 distribution of VDR SNPs alleles showed no significant difference between the present studied
210 subjects and the black race population. On the other hand, the white race population and Chinese
211 people showed significant differences in the allele frequencies of the VDR SNPs compared to the
212 present study as shown in Table 2.

213

214 **Table 2**

215

216 3.3. BLLs among the four VDR SNPs

217 Figure 1 shows the association between VDR SNPs and BLLs among the studied children. The *Apal*
218 polymorphism exhibited a higher mean BLL (25.9 µg/dL) in aa genotype and a lower BLL (16.9
219 µg/dL) in the Aa genotype ($\chi^2 = 11.3$, DF = 2, $p = 0.003$). No variations were observed in BLLs in
220 *TaqI*, *BsmI*, and *FokI* SNP genotypes (Fig. 2). Further, we examined the different VDR SNPs
221 genotype combinations and revealed the highest mean BLL (37.8 ± 14.9 µg/dL) in AA/TT/bb/Ff
222 combination (Table S1). The highest BLL (60.8 µg/dL) was also observed in this group (Fig. 2).

223

224 **Fig. 2.**

225 3.4. Association between VDR SNPs and susceptibility to lead toxicity

226 To examine the impact of BLLs on the VDR genotyping frequency and their association with risk
227 of lead toxicity, we categorized the subjects based on the lowest quartile of blood lead (Q1) into two
228 groups as low BLL children (BLL ≤ 10 $\mu\text{g/dL}$) and high BLL children (BLL > 10 $\mu\text{g/dL}$). Table 3
229 shows the associations of VDR SNPs genotype distributions with the risk of lead toxicity in low and
230 high BLL groups.

231

232 **Table 3**

233

234

235 Results showed that the only *Apal* genotype distributions were different in the two studied groups
236 ($\chi^2 = 6.68$, $p = 0.03$). Significantly higher aa genotype among high BLL children was found
237 compared to low BLL children. The frequencies of *Apal* genotypes determined from low BLL
238 children were 100% for (AA + Aa) and 0% for aa genotype while in high BLL children the frequency
239 of (AA + Aa) and aa was present in a percentage of 87% and 13%, respectively. No statistically
240 significant correlation was observed between the VDR genotypes and susceptibility to lead toxicity.
241 However, the BB+Bb (OR = 1.36), FF (OR = 1.07), AA (OR = 1.44) and TT (OR = 1.54) genotypes
242 showed an OR value of greater than 1 (Table 3). This result suggests the possibility of a higher risk
243 of lead toxicity to children with high frequencies of TT, AA, BB, and FF genotypes than the tt, aa,
244 bb, and ff genotypes carrier children, respectively.

245

246 Table 4 shows VDR genotype distributions and the risk of lead toxicity in association with gender.
247 Genotypic frequencies of 63% and 29% in females and 45% and 55% in males were observed for
248 TT and Tt genotypes, respectively. On the contrary, the tt genotype was found only in females with
249 a frequency of 8%. The *TaqI* genotype distribution between female and male subjects was
250 statistically different ($\chi^2 = 13.0, p = 0.001$). We found that the TT groups for *TaqI* SNP showed an
251 increased risk of lead toxicity in female children (OR = 2.06, 95% CI:1.04 – 4.06, $p = 0.03$). For the
252 other VDR SNPs, we did not observe a significant association but, an OR > 1 in the presence of one
253 or mixed alleles was observed.

254

255 **Table 4**

256 3.5. haplotype analysis of VDR genes

257 Haplotype analysis performed in the four-marker haplotype alleles (VDR-*FokI*, *BsmI*, *ApaI*, and
258 *TaqI*) was presented in Table 5. It was observed 10 haplotypes with a frequency above 1% in our
259 analysis. The three most represented haplotype were *FbaT* (28.7%), followed by *FbAT* (25.7%) and
260 *FbAt* (12.1%). Compared between the children, the frequency of *FbAt* was significantly higher in
261 low BLL children (21.9%) compared to children with high BLL (9.9%). The association analysis
262 between the haplotype and susceptibility to lead toxicity showed that the *FbAt* haplotype was
263 associated with a statistically decrease risk of lead toxicity (OR = 0.33, 95% CI:0.11 – 1.00, $p =$
264 0.04), indicating a protective role against lead toxicity.

265

266 **Table 5**

267 4. Discussion

268 Lead exposure can cause adverse health effects, particularly in children. To our knowledge, this is
269 the first study to investigate the ALAD and VDR genes SNPs in association with lead toxicity in
270 children living closer to or/and far away from an abandoned lead-zinc mine in Kabwe, Zambia.
271 Considering 5 µg/dL, the current CDC blood reference value, 92% of the children's blood samples
272 exceeded this value in the present study. Thus, the findings from this study may have some
273 implications for lead toxicity and support the ongoing efforts to reduce childhood lead exposure in
274 a region where only levels of lead in human and biota samples were investigated without any further
275 genetic analysis. Thus, in the current study, we aimed to assess the impact of lead exposure in
276 children through the biomarkers of susceptibility genes such as ALAD and VDR genes
277 polymorphisms ([Sakai, 2000a](#)).

278 In the current study, we found all children were ALAD 1-1 genotypes. This finding was in
279 accordance with other African population studies from Liberian populations ([Benkmann et al., 1983](#)),
280 Ghanaian, Ovambos (Bantusin of Namibia), and Xhosas (Cape Town of South Africa) ([Fujihara et
281 al., 2009](#)) that showed 100% ALAD 1-1 genotype, with neither ALAD 1-2 nor ALAD 2-2 genotypes.
282 Although the more electronegative ALAD 2 allele could bind more lead easily and maintain higher
283 BLLs than the ALAD 1 allele, the latter allele could be more prone to the detrimental effects of lead
284 because of the easy transfer of the bioavailable lead from blood to the soft and hard tissues ([Kelada
285 et al., 2001](#)). Previous studies reported that individuals with ALAD 1-1 genotypes had higher bone
286 lead levels, which suggested higher body burdens of lead and could be at greater risk of the long-
287 term health effects of lead ([Wan et al., 2014](#); [Yang et al., 2012](#)). [Kim et al. \(2004\)](#) also reported
288 higher hematological effects of lead for ALAD 1 homozygote subjects and they are more likely to
289 be anemic. Another study on male Japanese lead workers by [Sakai et al. \(2000b\)](#) showed high

290 aminolevulinic acid (ALA), which is neurotoxic to the brain, in plasma for ALAD 1-1 genotype.
291 These results suggest that the ALAD 1 homozygotes may be at greater risk due to the bioavailable
292 lead in the blood that can transfer and accumulate in the tissues and brain. Also, we reported
293 significant hypermethylation of the ALAD gene in these lead-exposed children with an odd ratio
294 of greater than one for anemia (Yohannes et al., 2020). Thus, the people, particularly infants and
295 children in Kabwe might be at high risk of lead poisoning.

296 The genetic variants of VDR have been identified as potential factors to influence the lead
297 toxicokinetic in the human body, and the effects of VDR SNPs such as *FokI*, *BsmI*, *ApaI*, and *TaqI*
298 have been reported (Onalaja and Claudio, 2000; Rezende et al., 2008). In the present study, the
299 observed genotype frequencies of the four VDR SNPs are conformed to the Hardy-Weinberg
300 equilibrium. The A, b, F, and T alleles were the dominant alleles with incidences of 68%, 80%, 81%,
301 and 75% in *ApaI*, *BsmI*, *FokI*, and *TaqI* polymorphisms, respectively. The most common genotype
302 for each polymorphism was bb (62.1%) for *BsmI*, FF (65.7%) for *FokI*, both AA and Aa (45%) for
303 *ApaI*, and TT (53.6%) for *TaqI*. The general distributions of these VDR SNPs were consistent with
304 other population studies of black South Africans (Meyer et al., 2017), African Americans
305 (Sarkissyan et al., 2014), and African ancestry studies (Lins et al., 2011). On the contrary, there were
306 significant differences in allele distribution between this study subjects and white South Africans
307 (Meyer et al., 2017), European ancestry, Asian ancestry and Brazilians (Lins et al., 2011), and
308 Chinese population (Yu et al., 2017). So, our results offered additional evidence to the distribution
309 specificity of VDR gene polymorphisms based on ethnicity.

310 In the present study, we found an association of high BLLs in *ApaI* polymorphism variants. The
311 prevalence of aa genotype increases from 0% (Q1) to 22% (Q4), and the aa genotype of *ApaI* variants
312 had significantly increased mean blood lead levels than those of the other genotypes (aa vs AA, $p =$

313 0.02; aa vs Aa $p = 0.001$). This finding corroborates with previous studies reported that *ApaI* aa
314 genotype had higher BLLs than the Aa and AA genotypes in workers exposed to lead due to their
315 workplace (Chuang et al., 2004; Himani et al., 2020). Further analysis among the SNPs showed that
316 the aa genotype had significantly higher BLL than FF, Ff, Bb, bb, and Tt genotypes ($p < 0.05$). No
317 significant associations of BLLs within *BsmI*, *FokI*, and *TaqI* variants were observed.

318 The influences of VDR SNPs to different traits have been investigated in adults with occupational
319 lead exposure (Chuang et al., 2004; Himani et al., 2020; Shaik et al., 2019; Wananukul et al., 2012)
320 or patients with different types of diseases such as cancer (Köstner et al., 2009), diabetes (Ahmed et
321 al., 2019) and autism (Cieślińska et al., 2017). In the current study, we investigated the correlation
322 between the VDR SNPs and environmental lead exposure in children. The results showed no
323 statistical associations between the genotypes at the VDR polymorphic sites and susceptibility to
324 lead toxicity. Nevertheless, the BB+Bb, FF, AA, and TT genotypes showed OR values of greater
325 than one, but not statistically significant. Previous studies showed the association of these VDR
326 genotypes with bone mineral density. Haynes et al. (2003) showed increased bone density in the
327 VDR-*FokI* FF genotype while the AA genotype in *ApaI* and BB genotype in *BsmI* were associated
328 with lower bone mineral density (Li et al., 2012). In another study by Pawlas et al. (2012), the *BsmI*
329 B allele showed an inverse correlation against cognitive function with a steeper slope ($\beta = -0.08$),
330 suggesting that lead has a negative influence on children's performance IQ. In the present study, the
331 highest BLL (60.8 $\mu\text{g/dL}$) was detected in these genotypes. Thus, children with high frequencies of
332 those genotypes might be at higher risk of calcium absorption and IQ problem. We also found an
333 association between gender and *TaqI* polymorphism. In the present study, female children with TT
334 genotypes showed higher susceptibility to lead toxicity (OR = 2.06, $p = 0.03$) than males.
335 Chakraborty and his coworkers (2008) examined the effect of lead on the neuromotor response in

336 association with *TaqI* genotypes in 82 children and observed adverse effects of lead exposure on
337 postural balance response in association with genetic polymorphisms of VDR at the *TaqI* site. A
338 study from the National Health and Nutrition Examination Survey also investigated the relationship
339 between the children's cognitive function and blood lead concentration in association with VDR
340 SNPs and suggested that the *TaqI* variants can alter the children's central nervous system toxicity of
341 lead (Krieg Jr et al., 2010). These results suggest that the children in Kabwe might be at higher risk
342 for the biological effects of lead. In the current study, haplotype structure revealed 10 haplotypes
343 with >1% frequency. There was a trend of increased frequency of *FbAt* haplotype in low BLL
344 children compared to High BLL children, suggesting that this haplotype could be protective against
345 lead toxicity.

346 There are limitations to our study. First, this study is a cross-sectional study that collected samples
347 at a single point in time, and thus it is difficult to determine incidence. Second, the small sample size
348 may weaken the statistical power. Third, the current study depends on lead levels using blood
349 samples at which the half-life of lead is about 35 days in blood, which may inevitably affect the
350 result to some degree. Reliable bone lead measurements are needed. Thus, our findings need to be
351 replicated in other long-term follow-up studies with large scale samples among the population
352 exposed to both environmental and occupational lead, and bone lead measurement with the
353 prevalence of disease as VDR SNPs have been linked to human traits.

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358 **5. Conclusions**

359 This study provides insight into the association of ALAD and VDR polymorphic variants in
360 association with BLL in children exposed to environmental lead. Our finding suggests that all
361 subjects were ALAD 1 homozygotes at which they might be at risk due to the bioavailable lead in
362 the blood that can transit to soft tissues and the brain. Regarding the VDR SNPs, the *Apal*
363 homozygous mutant genotype showed association with BLLs in environmentally lead-exposed
364 children. The TT-genotype in *TaqI* (rs731236) site was allied with lead toxicity in female children.
365 In haplotype analysis, the haplotype *FbAt* showed a protective role against lead toxicity compared
366 to the most frequent haplotype (*FbaT*). Overall, further larger-scale samples alongside bone lead
367 measurements are warranted to assess the ALAD and VDR SNPs in association with their
368 implications on lead toxicity manifestations in the general population.

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370

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375

376 **Declaration of Competing Interest:**

377 None.

378

379 **Authors Contribution**

380 **Yared B. Yohannes:** Conceptualization, Data curation, Formal analysis, Methodology, Writing -
381 original draft, Writing - review & editing; **Shouta M.M. Nakayama** and **Yoshinori Ikenaka:**
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383 **Haruya Toyomaki, Andrew Kataba** and **Kaampwe Muzandu:** Investigation, Writing - review
384 & editing; **Kennedy Choongo:** Supervision; **Mayumi Ishizuka:** Funding acquisition, Project
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397

398 **Ethical approval**

399 The study protocol was approved by the University of Zambia Research Ethics Committee
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Tables and Figures

Figure captions

Fig 1. PCR-RFLP analysis for VDR gene- FokI, BsmI, ApaI and TaqI polymorphisms. M: 100 bp DNA marker; Lane 1: ff homozygote, Lane 2: Ff heterozygote, Lane 3: FF homozygote; Lane 4: bb homozygote, Lane 5: Bb heterozygote, Lane 6: BB homozygote; Lane 7: aa homozygote, Lane 8: Aa heterozygote, Lane 9: AA homozygote; Lane 10: tt homozygote, Lane 11: Tt heterozygote, Lane 12: TT homozygote.

Fig 2. Blood lead levels for all genotypes of VDR gene polymorphic variants (*BsmI*, *FokI*, *ApaI* and *TaqI*) in environmental lead exposed children (* = $p < 0.05$; ** = $p < 0.01$; nonparametric Wilcoxon / Kruskal-Wallis tests between aa genotype and other genotypes)

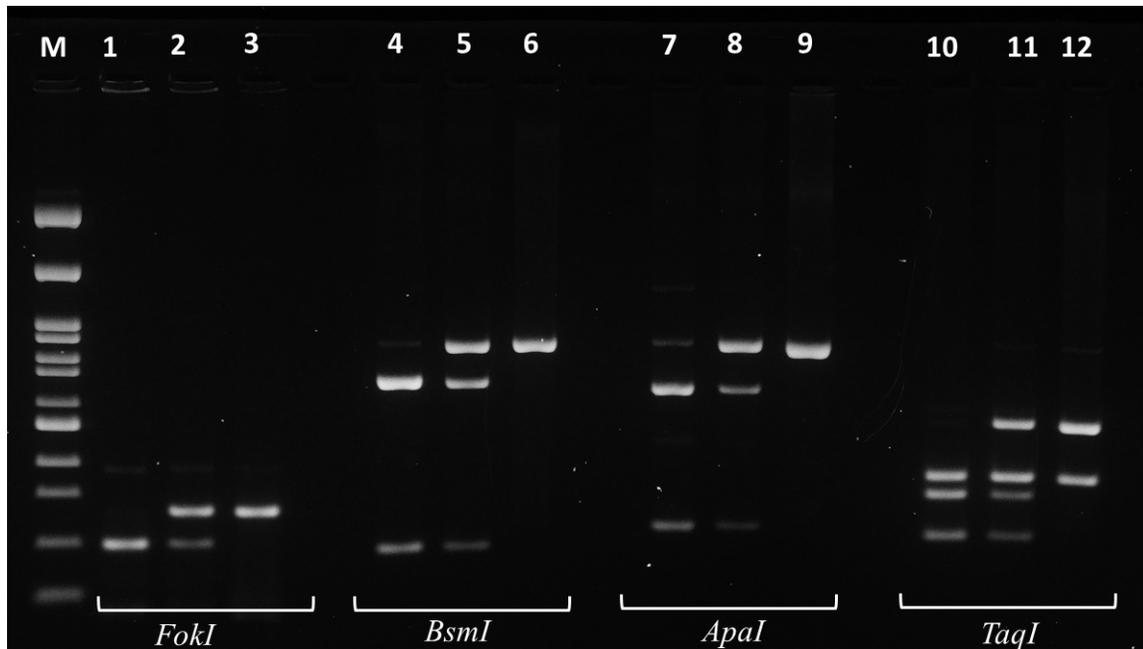


Fig 1.

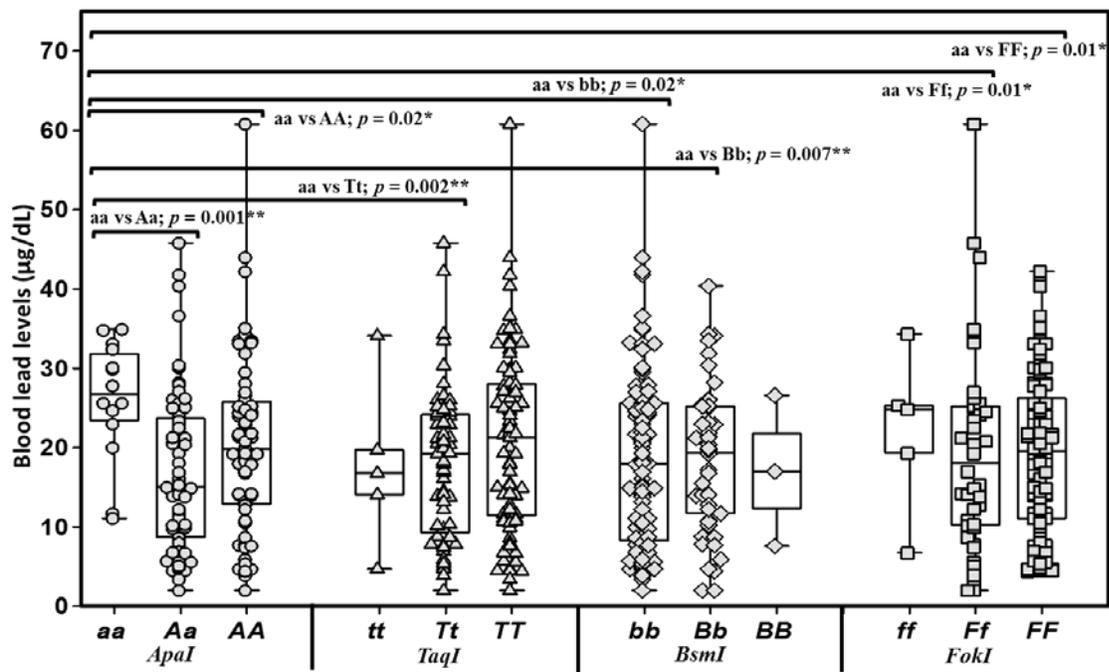


Fig 2.

Table 1: Genotype and allele frequency of ALAD and VDR polymorphisms (*BsmI*, *FokI*, *ApaI* and *TaqI*) in children from Kabwe, Zambia in comparison with quartiles of BLLs

Gene	Genotype	N (%)	HWE	Quartiles of BLL ^b				<i>p</i> -Value *
			equation ^a	Q1, N (%)	Q2, N (%)	Q3, N (%)	Q4, N (%)	
ALAD	ALAD 1-1	140 (100)		31 (22.1)	36 (25.7)	32 (22.9)	41 (29.3)	
	ALAD 1-2	0						
	ALAD 2-2	0						
	G	1						
	C	0						
<i>FokI</i> T > C	FF (CC)	92 (65.7)	0.001, 0.99	20 (64.5)	24 (66.7)	21 (65.6)	27 (65.8)	NS
	Ff (CT)	43 (30.7)		10 (32.3)	12 (33.3)	9 (28.1)	12 (29.3)	
	ff (TT)	5 (3.6)		1 (3.2)	0	2 (6.3)	2 (4.9)	
	F (C)	81						
	f (T)	19						
<i>BsmI</i> G > A	BB (AA)	3 (2.1)	1.88, 0.17	1 (3.2)	1 (2.8)	0	1 (2.4)	NS
	Bb (AG)	50 (35.7)		9 (29.1)	14 (38.9)	13 (40.6)	14 (34.1)	
	bb (GG)	87 (62.1)		21 (67.7)	21 (58.3)	19 (59.4)	26 (63.4)	
	B (A)	20						
	b (G)	80						
<i>ApaI</i> G > T	AA (TT)	63 (45.0)	0.09, 0.76	12 (38.7)	16 (44.4)	17 (53.1)	18 (43.9)	0.01*
	Aa (TG)	63 (45.0)		19 (61.3)	18 (50.0)	12 (37.5)	14 (34.1)	
	aa (GG)	14 (10.0)		0	2 (5.6)	3 (9.4)	9 (22.0)	
	A (T)	68		69	69	72	61	
	a (G)	32		31	31	28	39	
<i>TaqI</i> T > C	TT (TT)	75 (53.6)	2.85, 0.09	14 (45.2)	20 (55.6)	13 (40.6)	28 (68.3)	NS
	Tt (TC)	60 (42.8)		16 (51.6)	14 (38.9)	18 (56.3)	12 (29.3)	
	tt (CC)	5 (3.6)		1 (3.2)	2 (5.5)	1 (3.1)	1 (2.4)	
	T (T)	75						
	t (C)	25						

N: number; HWE: Hardy-Weinberg equilibrium

* Chi-square test

^a HWE equation: χ^2 (chi-squared test) < 3.841 and/or *p*-value > 0.05 indicates no deviation from HWE

^b BLL quartiles (Q1: $x \leq 10 \mu\text{g/dL}$; Q2: $10 < x \leq 19 \mu\text{g/dL}$; Q3: $19 < x \leq 25 \mu\text{g/dL}$; Q4: $x > 25 \mu\text{g/dL}$)

Table 2: Comparison of VDR SNPs allele frequencies between Zambian children and different populations

Population	<i>FokI</i> (rs2228570)	<i>BsmI</i> (rs1544410)	<i>Apal</i> (rs7975232)	<i>TaqI</i> (rs731236)	Reference
	C/T	G/A	T/G	T/C	
Zambian children	0.81/0.19	0.80/0.20	0.68/0.32	0.75/0.25	This study
African ancestry	0.83/0.17	0.71/0.29	0.62/0.38	0.75/0.25	Lins et al., 2011
European ancestry	0.52/0.48***	0.52/0.48***	0.57/0.43	0.52/0.48**	"
Asian ancestry	0.64/0.36**	0.92/0.08**	0.35/0.65***	0.93/0.07***	"
Brazil	0.67/0.33*	0.60/0.40**	0.54/0.46*	0.62/0.38	"
Black South Africans	0.84/0.16	0.74/0.26	0.76/0.24	0.66/0.34	Meyer et al., 2017
White South Africans	0.54/0.46***	0.62/0.38**	0.54/0.46*	0.61/0.39*	"
African American	0.77/0.23	0.63/0.37**	0.70/0.30	0.73/0.27	Sarkissyan et al., 2014
China	0.60/0.40**	0.91/0.08**	0.35/0.65***	0.90/0.10***	Yu et al., 2017
* = p < 0.05; ** = p < 0.01; *** = p < 0.001; Two-tailed Fisher's exact test and chi square test was done using Zambian children as reference value					

Table 3: Distribution of VDR genotypes in low vs high BLL subjects and associations with lead toxicity

SNP	Genotype / Allele	High BLL children [#] (N = 109)		Low BLL children [#] (N = 31)		HWE equation χ^2, p	OR [95% CI], <i>p</i> -value
		N	%	N	%		
BsmI	BB	2	1.8	1	3.2	0.91, 0.63	(BB + Bb) vs bb 1.36 [0.58 – 3.18], 0.46
	Bb	41	37.6	9	29		
	bb	66	60.6	21	67.7		
FokI	FF	72	66.0	20	64.5	0.05, 0.97	FF vs (Ff + ff) 1.07 [0.46 – 2.46], 0.87
	Ff	33	30.3	10	32.3		
	ff	4	3.7	1	3.2		
ApaI	AA	52	46.8	12	38.7	6.68, 0.03*	AA vs (Aa + aa) 1.44 [0.64 – 3.26], 0.37
	Aa	43	40.4	19	61.3		
	aa	14	12.8	0	0		
TaqI	TT	61	56.0	14	45.2	1.25, 0.54	TT vs (Tt + tt) 1.54 [0.69 – 3.44], 0.28
	Tt	44	40.4	16	51.6		
	tt	4	3.6	1	3.2		

N: Number of children; %: Frequency; BLL: Blood lead level; HWE: Hardy-Weinberg equilibrium
 OR: Odds ratios; 95% CI: 95% Confidence interval
[#] Low BLL children: BLL ≤ 10 µg/dL; High BLL children: BLL > 10 µg/dL
 * Result is significant at *p* < 0.05

Table 4: Genotype frequencies of VDR SNPs and association with gender

SNP/ Genotype	All children		HWE equation χ^2, p	OR [95% CI], <i>p</i> -value Female vs Male
	Female N (%)	Male N (%)		
<i>BsmI</i>	BB	1 (1.5)	2 (3)	0.26, 0.87 (BB + Bb) vs bb 1.04 [0.54 – 2.08], 0.89
	Bb	24 (37)	26 (34)	
	bb	40 (61.5)	47 (63)	
<i>FokI</i>	FF	44 (68)	48 (64)	4.51, 0.11 FF vs (Ff + ff) 1.17 [0.58 – 2.38], 0.64
	Ff	21 (32)	22 (29)	
	ff	0 (0)	5 (7)	
<i>ApaI</i>	AA	29 (45)	34 (45)	0.11, 0.94 AA vs (Aa + aa) 0.92 [0.47 – 1.79], 0.81
	Aa	30 (46)	33 (44)	
	aa	6 (9)	8 (11)	
<i>TaqI</i>	TT	41 (63)	34 (45)	13.0, 0.001** TT vs (Tt + tt) 2.06 [1.04 – 4.06], 0.03*
	Tt	19 (29)	41 (55)	
	tt	5 (8)	0 (0)	

N: Number of children; %: Frequency; HWE: Hardy-Weinberg equilibrium
 OR: Odds ratios; 95% CI: 95% Confidence interval
 * Result is significant at *p* < 0.05

Table 5: Haplotypes frequency of vitamin D receptor gene polymorphisms in high and low BLL children

<i>FokI</i>	<i>BsmI</i>	<i>ApaI</i>	<i>TaqI</i>	Total	High BLL children [#]	Low BLL children [#]	OR (95% CI)
<i>F</i>	<i>b</i>	<i>a</i>	<i>T</i>	0.287	0.295	0.258	1
<i>F</i>	<i>b</i>	<i>A</i>	<i>T</i>	0.257	0.274	0.188	1.58 (0.60 - 4.13)
<i>F</i>	<i>b</i>	<i>A</i>	<i>t</i>	0.121	0.099	0.219	0.33 (0.11 - 1.00) *
<i>F</i>	<i>B</i>	<i>A</i>	<i>T</i>	0.083	0.079	0.069	1.29 (0.29 - 5.68)
<i>f</i>	<i>b</i>	<i>A</i>	<i>T</i>	0.071	0.064	0.108	0.41 (0.10 - 1.61)
<i>F</i>	<i>B</i>	<i>A</i>	<i>t</i>	0.065	0.068	0.071	0.76 (0.17 - 3.46)
<i>f</i>	<i>b</i>	<i>a</i>	<i>T</i>	0.034	0.030	0.048	0.73 (0.10 - 5.37)
<i>f</i>	<i>B</i>	<i>A</i>	<i>T</i>	0.030	0.036	0.036	0.62 (0.06 - 6.19)
<i>f</i>	<i>b</i>	<i>A</i>	<i>t</i>	0.028	0.029	0	
<i>f</i>	<i>B</i>	<i>A</i>	<i>t</i>	0.020	0.022	0	

[#] Low BLL children: BLL ≤ 10 µg/dL; High BLL children: BLL > 10 µg/dL
* Result is significant at $p < 0.05$