Evaluation of Elements in Hair Samples of Children with Developmental Language Disorder (DLD)

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ABSTRACT

BACKGROUND: Recent studies have highlighted a role for trace trace elements and toxic metals across neurodevelopmental disorders including developmental stuttering, Autistic Spectrum Disorders (ASD) and Attention Deficit/Hyperactivity Disorder (ADHD). However, these environmental influences have yet to be explored in relation to Developmental Language Disorder (DLD).

METHODS: Elemental hair composition of 7 elements; zinc ($^{64}\text{Zn}$), magnesium ($^{26}\text{Mg}$), iron ($^{57}\text{Fe}$), potassium ($^{39}\text{K}$), aluminum ($^{27}\text{Al}$), lead ($^{208}\text{Pb}$), and barium ($^{138}\text{Ba}$) were analyzed in hair samples from 35 children affected by DLD and 35 controls with typical language development (TLD) using both inductive coupled plasma optical emission spectroscopy (ICP–OES) and inductive coupled plasma mass spectroscopy (ICP–MS).

RESULTS: The concentration of $^{64}\text{Zn}$ was significantly lower in the hair of DLD group compared to the TLD control group. All other elements showed similar levels between cases and controls. This pilot study demonstrates the utility of trace elements and toxic metals screening in relation to language disorders and the use of hair samples in such investigations.

CONCLUSION: The finding that zinc levels differed between cases and controls could represent a clinically relevant result and should be replicated in a larger sample size across time. A wider battery of related elements will help to better understand the role of trace elements and toxic metals in DLD.

Key words: Developmental language disorder; Metals; Human hair; ICP-MS; ICP-OES
1. Introduction

Language development is a fundamental part of child development, and an essential primary step in learning. It supports both verbal and non-verbal communication, problem solving and intellectual skills and social relationships. Delays in language acquisition milestones are associated with reduced educational and emotional functioning [1]. Children with persistent language difficulties have poorer long-term outcomes in terms of quality of life, educational attainment and employment opportunities [2-4].

A recent population study of over 7000 children in the UK estimated that 9.92% of children have language disorder at school entry (aged 4-5 years) [5]. Three quarters of these children, or 7.58% of the total population sampled, had no identifiable medical cause for their language difficulties; in which case, the impairment is known as Developmental Language Disorder (DLD), or Specific Language Impairment (SLI) [5]. These two terms are often used interchangeably according to the country and setting where the diagnosis was made. Technically, Specific Language Impairment has stricter non-verbal IQ thresholds and medical exclusion criteria [6].

The prevalence of language delay in India was 6% among children between 0 and 36 months [7]. The rate among children in kindergarten (5–6 years) were reported as 12.6% by Beitchman et al. [8] and 7.4% by Tomblin et al. [9] in large-scale epidemiological studies in Canada and the US, respectively. The prevalence among 3-year-old children in New Zealand/Dunedin was 7.6% [10]. In the Arab world, similar percentages of prevalence have been expected although fewer studies have been conducted. For example, screening of DLD children at 3 years of age in the United Arab Emirates (UAE) indicated that 9.9% were found to have delays in the language
sector of the Denver Developmental Screening Test and 6.5% as having general language disability [11].

Despite wide-ranging research, the causes of DLD/SLI remain unknown. The disorder tends to run in families, where it co-occurs with other neurodevelopmental disorders such as dyslexia and ADHD [12, 13]. Language ability and language disorders, particularly those that affect speech production are both reported to have a significant heritable component [14] but there are also significant shared environmental effects, particularly at very young ages [15]. Most genetic investigations conclude that, in the majority of cases, DLD is a complex disorder involving complex interactions between many risk factors, both genetic and environmental. It is a heterogeneous disorder that have no apparent motoric etiology and includes deficits in both expressive language (e.g., grammar, syntax, and semantics) as well as receptive language. This heterogeneity of DLD obstructs accurate evaluation, effective treatment protocols, and causes problems in the identification of causal factors. [16].

Bioelements are organic compounds that form the basic components of vitamins, proteins, and enzymes and play a vital role in human cell metabolism [17]. Exposure to these elements form part of a shared environment, or the so-called exposome, and have been shown to influence neurodevelopmental outcomes when exposure is in utero [18] or neonatal [19, 20]. The most common bioelements are hydrogen, oxygen, carbon, nitrogen and phosphorus, which together make up 96% of the total body weight [21]. Other trace bioelements such as potassium, zinc, magnesium and iron make up a far smaller proportion of living matter but, nonetheless, are essential to cellular function. These elements act as cofactors for enzyme function across a broad range of physiological processes [21]. Other metalloid trace elements such as lead, aluminium
and barium, have little nutritional value or biological function but are nonetheless found in cells and can act as biomarkers of environmental exposure [22].

The neurotoxic effects of extreme levels (excess of deficiency) to trace elements is well documented [23] but, because the natural levels of the elements are so low, more subtle imbalances, that fall outside of malnutrition, can be hard to detect. Because these elements are involved in a wide-range of essential catalytic reactions, the effects of imbalance can be widespread. Recent technological developments allow the exact measurement of these bioelements across a large number of samples allowing links to be made between trace element imbalances and phenotypic states.

Of particular relevance to this paper, current literature highlights differences across a range of metal and essential bioelement levels in children affected by neurodevelopmental disorders, predominantly in Attention Deficit Hyperactivity Disorder (ADHD) and Autistic Spectrum Disorder (ASD). Significantly higher levels of heavy metals (mercury, lead, arsenic, antimony and cadmium), and lower levels of essentials trace elements (calcium, copper, chromium, manganese and iron) were demonstrated in ASDS cases [24]. In another study by Arora et al., [25], higher levels of lead were observed over the prenatal period and first 5 months postnatally, Zinc levels were lower in cases during the third trimester, while constant lower levels of manganese levels were found both pre- and postnatally. Prenatal and early postnatal metal exposures were measured in ASD cases. Children with ASD showed lower prenatal and postnatal Copper (Cu) and prenatal Nickel concentrations and Copper-to-Zinc (Cu/Zn) ratio as compared with control children. Prenatal Cu exposure and Cu/Zn ratio were found to be positively correlated with Language and communication scores in children with ASD. The findings of this study suggest that prenatal exposure to some metals may be important for neurodevelopment in children with ASD [26].
The clinical utility of ADHD using supplementation of the numerous elements including zinc alone or in mixture with iron as well as magnesium has yet to be demonstrated. [30]. The literature thus emphasizes the need for high-quality and well-controlled studies to validate potential links [30, 31, 32]. Beyond ASD and ADHD, bioelement levels have been shown to correlate with more general features of neurodevelopment; lead levels in umbilical cord samples have been shown to negatively correlate with sociability outcomes in young infants [33] and maternal magnesium and plasma zinc levels have been marginally associated with school readiness [34] and motor and language abilities at 1 year of age [35]. However the field has yet to assess the role of such elements in relation to Developmental Language Disorders (DLD).

In this pilot study, we perform trace elements and toxic metals screen in hair samples of children with DLD compared to those with typical language development. We determine the concentration of trace elements (zinc (\(^{64}\text{Zn}\)), magnesium (\(^{26}\text{Mg}\)), iron (\(^{57}\text{Fe}\)), potassium (\(^{39}\text{K}\)), barium (\(^{138}\text{Ba}\)) and toxic metals (aluminum (\(^{27}\text{Al}\)) and lead (\(^{208}\text{Pb}\))) using two different instruments; inductive coupled plasma optical emission spectroscopy (ICP–OES) and inductive coupled plasma mass spectroscopy (ICP–MS). This study forms a baseline screen of common trace elements and toxic metals, with regard to language development.
Materials and Methods

2.1 Study design

The study was approved by the institution review board at Jordan University of Science and Technology (approval number 10/215/2444) and written informed consents were collected from all guardians.

This is a case-control study included 35 children with DLD (cases) who visited Speech Clinic and 35 children with typical language development (control) who visited the Paediatric Clinic at King Abdullah University Hospital (KAUH) in Jordan. Both cases and controls were gender and age matched ranging in age from 3-7 years. The DLD cases were 25 boys, and 10 girls (M = 5, SD= 2). The children with typical language development were 24 boys, and 11 girls (M = 4, SD= 2, boys=24, girls = 11).

All participants lived in the Northern Jordan area. All children were assessed by the same speech-language pathologist at the hospital using standardised test batteries of (Clinical Evaluation of Language Fundamentals-4, Peabody Picture Vocabulary-3, Goldman-Fristoe Test of Articulation-2). Audiological assessment including pure tone audiometry was performed to exclude any participant with hearing loss (average pure-tone thresholds above 25dBHL across frequencies of 500 Hz, 1000 Hz and 2000 Hz).

Any children who received dietary supplements or whose parents worked in a chemical factory were excluded from the study. Information regarding place of residence, parent occupation, parent smoking status and brand of shampoo used was collected by questionnaire from parents at time of hair sampling.

2.2 Hair sample collection
A piece of hair (2g) was cut adjacent to scalp from the back of the head using ethanol-clean scissors [31]. Collection was made by guardians after the child’s hair had been washed at least two times to remove any external substances. Hair sample was collected once from each participant and splitted into two specimens for later analyses.

2.3 Stock Materials

Acetone, hydrogen peroxide (H₂O₂), and Triton X 100 were purchased from Sigma Aldrich (St. Louis, MO, USA). Concentrated nitric acid (HNO₃) for trace element analysis was purchased from Scharlau, S.L. (Sentmenat, Barcelona, Spain). Multi-element stock solution of trace elements and toxic metals (Al, Ba, Fe, K, Mg, Pb, and Zn) were obtained from Accu Standards (Ashburn, Virginia, USA). Certified human hair (GBW0 7601a) was purchased from institute of geophysical and geochemical exploration (Langfang, Hebei, China).

2.4 Hair sample treatment

Hair samples were cleaned by soaking in Triton X100 and acetone solution for 5 min. followed by an immediate rinsing with DI water. Once the water had evaporated, the hair was dried and kept at –20 °C. Subsequently, hair was then frozen in liquid nitrogen and ground to a fine powder using a grinder [36]. Duplicate subsamples of 200mg of powdered hair were digested using 8ml of HNO₃ and 2ml of H₂O₂ in a microwave digestive system. Afterwards, digested samples were heated on hot plate at 90 °C for 45 minutes [36]. Three subsamples of certified hair powder were analyzed randomly within the study samples.

2.5 Instrumentation

Two instruments were used to measure 5 trace elements (Ba, Fe, K, Mg, and Zn) and 2 toxic metals (Al, Pb); the iCAPQ Inductively Coupled Plasma Mass Spectrometer
(Thermo Fisher Scientific, Darmstadt, Germany) (ICP-MS) at Jordan University of Science and Technology and the iCAP-6300 radial view Inductively Coupled Plasma Optical Emission (Thermo Fisher Scientific, Waltham, MA, USA) (ICP–OES) at Jordan Atomic Energy Commission. Darmstadt software was used in the data analysis of both sample sets [37]. Each sample was analyzed one month apart on each instrument in a random manner.

The elements selected for the purpose of the current study were based on preliminary chemical analyses on both instrument (ICP-MS and ICP-OES). The elements that measured within limit of detection and limit of quantification for both instruments were included in the current study.

The wavelengths of measured elements are presented in Table 1.

**Table 1** Wavelength of studied elements.

<table>
<thead>
<tr>
<th>Element</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{64}\text{Zn}$</td>
<td>206.2</td>
</tr>
<tr>
<td>$^{26}\text{Mg}$</td>
<td>279.6</td>
</tr>
<tr>
<td>$^{57}\text{Fe}$</td>
<td>240.5</td>
</tr>
<tr>
<td>$^{39}\text{K}$</td>
<td>766.5</td>
</tr>
<tr>
<td>$^{27}\text{Al}$</td>
<td>308.2</td>
</tr>
<tr>
<td>$^{208}\text{Pb}$</td>
<td>220.4</td>
</tr>
<tr>
<td>$^{138}\text{Ba}$</td>
<td>230.4</td>
</tr>
</tbody>
</table>

2.6 *Calibration*

The 7 calibration curves were created using five different concentrations (5, 10, 50, 100, 500 ppm) prepared from the multi-element standard stock solutions. Standard and
blank solutions were prepared in 2% trace analysis grade HNO₃. The regression coefficient (R²) of all the calibration curves was higher than 0.99 from both instruments.

2.7 Statistical analysis
Statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 18 (IBM, Armonk, NY, USA). Descriptive statistics were calculated in case and control groups. One-way analysis of variance (ANOVA) was performed to compare the mean concentrations of elements in hair samples of cases and controls. ANOVA was used to find which elements have the largest F value which is the ratio between group variance and within group variance. The data were proposed as mean ± standard deviation (M ± SD). The p value ≤ 0.05 was considered significant for all tests.
3. Results

The accuracy of the method was calculated using certified human hair of identified elemental content. Table 2 represents comparisons between certified values and measured values of 7 elements by the two instruments. The relative errors were calculated using the following equation:

\[
\text{Relative error} = \frac{\text{Measured value} - \text{Assigned value}}{\text{Assigned value}} \times 100\%
\]

Based on the presented results, the measurements of elemental hair composition using either ICP-OES or ICP-MS are accurate for 7 investigated elements of average relative error 7%.

Table 2 The mean and standard deviation of certified and measured concentrations (ppm) of certified hair samples.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Assigned value (ppm)</th>
<th>Measured value by ICP-OES (ppm)</th>
<th>Relative error %</th>
<th>Measured value by ICP-MS (ppm)</th>
<th>Relative error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{64}\text{Zn})</td>
<td>137±9</td>
<td>129±7</td>
<td>6</td>
<td>134±5</td>
<td>2</td>
</tr>
<tr>
<td>(^{26}\text{Mg})</td>
<td>140±10</td>
<td>133±2</td>
<td>5</td>
<td>137±18</td>
<td>2</td>
</tr>
<tr>
<td>(^{57}\text{Fe})</td>
<td>36±5</td>
<td>37±3</td>
<td>3</td>
<td>34.7±5.9</td>
<td>2</td>
</tr>
<tr>
<td>(^{39}\text{K})</td>
<td>44±13</td>
<td>43±11</td>
<td>2</td>
<td>43.8±10</td>
<td>1</td>
</tr>
<tr>
<td>(^{27}\text{Al})</td>
<td>1100±100</td>
<td>1316±73</td>
<td>20</td>
<td>1377±50</td>
<td>25</td>
</tr>
<tr>
<td>(^{208}\text{Pb})</td>
<td>5.7±0.5</td>
<td>6.9±2.7</td>
<td>5</td>
<td>6.3±0.4</td>
<td>11</td>
</tr>
<tr>
<td>(^{138}\text{Ba})</td>
<td>11.4±0.6</td>
<td>11.2±0.4</td>
<td>2</td>
<td>9.8±1.1</td>
<td>14</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>6</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>
A total of 70 samples were analysed from 35 DLD cases and 35 TLD controls aged between 3 and 7 years (Table 3).

Table 3 Demographic data of the two groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>n</th>
<th>Age (M±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLD</td>
<td>Male</td>
<td>25</td>
<td>5±2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>TLD</td>
<td>Male</td>
<td>24</td>
<td>4±2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

The number of samples per age is represented in Figure 1.

Figure 1: The number of samples per age (DLD cases).
One-way analysis of variance (ANOVA) was performed to compare the concentrations of studied elements in the hair samples of children with DLD and children with typical language development across both instruments (Table 4).

**Table 4**  Mean Concentrations, *F* value, and *P* value of elements determined by ICP-MS and ICP-OES, in cases and controls

<table>
<thead>
<tr>
<th>Element</th>
<th>ICP-MS</th>
<th></th>
<th>ICP-OES</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLD (n=35)</td>
<td>TLD (n=35)</td>
<td><em>F</em> value</td>
<td><em>P</em> value</td>
</tr>
<tr>
<td>64Zn</td>
<td>135±58</td>
<td>200±149</td>
<td>5</td>
<td>0.04</td>
</tr>
<tr>
<td>26Mg</td>
<td>131±13</td>
<td>139±145</td>
<td>0.045</td>
<td>0.8</td>
</tr>
<tr>
<td>57Fe</td>
<td>84±46</td>
<td>88±73</td>
<td>0.06</td>
<td>0.8</td>
</tr>
<tr>
<td>39K</td>
<td>93±54</td>
<td>85±56</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>27Al</td>
<td>28±15</td>
<td>27±20</td>
<td>0.02</td>
<td>0.9</td>
</tr>
<tr>
<td>208Pb</td>
<td>12±14</td>
<td>16±30</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>138Ba</td>
<td>3±5</td>
<td>3±2</td>
<td>0.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Elements with a significant difference between groups are shown in bold.

The only element that was found to differ between cases and controls was zinc, which showed a marginally lower concentration in DLD cases compared to controls across both instruments (*P*=0.04, Table 2, Figure 2).
Figure 2: The mean concentration (ppm) of Zn in cases and controls measured by ICP-OES (The bars represent 95% confidence interval).

Because there are more males than females samples in the current study, the mean concentration of Zn between males and females in both case and control groups were represented in Figure 3.
Figure 3: The mean concentration (ppm) of Zn in cases and controls of males and females measured by ICP-OES (The bars represent 95% confidence interval).
2 Discussion

In this study, children with DLD were found to have similar levels of trace elements magnesium, iron, barium, potassium and toxic metals lead, aluminium compared to TLD controls at ages 3-7 years. The only element that was found to differ between DLD children and TLD controls was zinc (averages of 135±58ppm and 162±77ppm compared to 200±149ppm and 233±165ppm in controls). Zinc levels for both groups fell within reported reference ranges [38] but it is widely accepted that ranges are affected by factors such as age, sex, geographical region, diet, ethnicity and hair color. The children in this study were age-matched, had similar sex-ratios across groups, lived in the same geographical area and were filtered for overt environmental contaminants (such as passive smoking and chemical workers). The levels observed in the control group fit with our previous investigations of trace elements and toxic metals in other clinical groups from the same area [37] although other researchers have reported that dietary intake of zinc in Jordan is often below the recommended limits [39].

Both of the instruments used showed a similar trend in zinc levels between cases and controls and the direction of effect fits with previous studies which also report zinc levels to be reduced in Jordanian children affected by developmental stuttering [37] and ASD [40] and more widely in children affected by ASD [41, 42] and ADHD [43] across plasma, serum and hair samples. These studies hypothesize links between zinc regulation of dopamine transporters, which are a target of ADHD medication [43] or between zinc and mitochondrial function in children with ASD [26]. Although some studies demonstrate a positive effect of zinc supplements in these disorders [44] and mouse models thereof [45-47], others report null [48] findings with respect to ASD and negative effects of zinc supplements upon language and motor development in typically developing children [49-51] indicating that the exact levels of zinc need to be tightly
regulated during neurodevelopment. Overt zinc deficiencies in prenatal and neurodevelopmental periods are associated with decreased activity, increased emotional behavior and impaired memory and cognition [52] demonstrating the importance of this trace element to brain development. Although zinc levels have been associated with other neurodevelopmental disorders, to our knowledge, this is the first time that zinc has been associated specifically with language disorder.

An Association between zinc deficiency and Shank3 has been investigated in some previous Studies. Shank3 is part of the Shank family proteins localized to the postsynaptic density of excitatory synapses in the cortex and hippocampus [53]. They are main regulators of postsynaptic function that interrelates with many postsynaptic molecules such as actin cytoskeleton, glutamate receptors, and structural proteins [53, 54]. It was found that Shank3 is important for synaptic plasticity and the trans-synaptic connection between the reliability of presynaptic neurotransmitter release and postsynaptic responsiveness [55]. The sterile alpha motif (SAM) domain of shank3 is necessary for postsynaptic localization and fastening zinc, therefore altering zinc levels may control Shank3 function in dendritic spines. This assumption has been supported by finding that zinc is a strong regulator of shank3 function in hippocampus neurons of rats [55]. It was also suggested that Shank3 is a crucial factor of a zinc-sensitive signaling system, regulating the strength of synapses that may be weakened in people with autism spectrum disorders [55-57]. This discussion of the relationship between zinc deficiency and the weakness of synapses in people with ASD could explain the findings of the current study in which language delay is the main symptom of autism spectrum disorder.

Hair samples are collected in biobank cohorts [58] but some researchers have debated the utility of this sample type because the composition of hair can be altered
by external exposure to materials such as bleaches, hair dyes, and shampoos[59, 60]. Moreover, most companies of hair analysis have not validated their analytical methods by comparing hair elements against their standard references. These limitations were avoided in the current study by washing children’s hair using baby shampoo at least two times before cutting, excluding the participants who used to dye or bleach their hair, and by cleaning hair samples in Triton X 100 and acetone solution to remove any external substances [61]. Our data show that levels of trace elements and toxic metals were consistent across samples and instruments (both ICP-MS and ICP-OES) taken one-month apart. Results from the study demonstrated accurate measurements of elemental hair composition using either ICP-OES of average relative error of 6% or ICP-MS of average relative error of 8% for the 7 investigated elements. These results demonstrate the utility of hair samples in the spectral investigation of child trace elements and toxic metals. Hair samples are painless and simple to collect and thus, when collection is adequately controlled, these samples could provide a useful alternative sample for the evaluation of biomarkers in children.

The current study demonstrates that magnesium, iron, potassium, aluminium, lead and barium levels do not differ in children with DLD from those in children with typical language and further suggests a novel relationship between reduced zinc levels and DLD. These findings are preliminary and need further investigations in larger sample sizes but represent the utility of trace elements and toxic metals screening in language disorders. Although this methodology has been applied to other childhood conditions, it is not routinely used in relation to DLD. Such studies therefore provide a baseline for further investigations, even if they act only to rule out possible aetiologies that have been described in other neurodevelopmental disorders.
The root of the zinc imbalance observed here remains unclear, as do the cellular pathways affected by reduced zinc levels. However, studies such as this represent the first step in identifying possible environmental factors that influence language development. The finding that zinc levels are lower in children with DLD may help to direct our attention to the study of related elements not included in this pilot screen. For example, copper levels, are often correlated with zinc and have also been shown to be dysregulated in ASD subjects [48] but were not measured in the current study. Full screens and controlled analyses may yield biomarkers and act as a catalyst for more effective identification and treatment of language disorders.
**Author Contributions:** A.B. and M.A; methodology, A.B.; software, A.B.; validation, A.B. and M.A.; formal analysis, A.B.; M.A writing—original draft preparation, M.A., M.E.L., D.F.N and F.A; writing—review and editing, H.K and A.A; visualization, A.B.; supervision, M.A.; project administration. All authors have read and agreed to the published version of the manuscript.

**Key Points**

- The concentration of Zn was significantly lower in the hair of DLD group
- This study provides an accurate measurement of the levels of selected elements using both ICP-OES and ICP-MS instruments.
- No significant differences were found in Elemental hair levels of magnesium (Mg), iron (Fe), potassium (K), aluminum (Al), lead (Pb), and barium (Ba) in children affected by DLD

**Declarations**

**Funding Information**
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**Compliance with Ethical Standards**

**Conflict of Interest**
The authors declare that they have no conflict of interest.

**Research Involving Human Participants and/or Animals**

**Statement of Human Rights**
The research was conducted in accordance with the 1964 Helsinki Declaration. The protocol of the study was approved by King Abdullah University Hospital (KAUH) institution review board (IRB) [Approval No. 10/215/2444]. Moreover, verbal and informed consent was obtained from the parents of the examined children before inclusion into the study, and hair sampling was performed in the presence of parents.
• Consent to Participate
  Informed consent was obtained from the parents of all children enrolled in the study.

• Data Availability Statement (DAS)
  Relevant documentation or data are available upon request.
References


