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Blood Lead Level, Urinary Porphobilinogen and Serum Acetylcholine in Nigerian Children with Autism Spectrum Disorder: A Case-Control Study

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ABSTRACT

Purpose: Autism spectrum disorder (ASD), a common neurodevelopmental disorder characterized by communication and behavioral deficits, remains a subject of unknown etiology. However, the interplay of heavy metal (e.g., Lead) toxicity, which can disrupt the heme synthesis pathway and lead to elevated metabolites like porphobilinogen (PBG), and altered neurotransmission have been implicated in the development of ASD. Therefore, this study examined blood lead level (BLL), serum acetylcholine (ACh), and urine PBG levels in Nigerian children with ASD. Methods: Forty seven participants aged 3 to 12 years were recruited, including 16 children diagnosed with ASD (cases), 16 children with neurodevelopmental disorders (NDDs) excluding ASD (positive controls), and 15 neurotypical children (negative controls). BLL was measured using atomic absorption spectroscopy (AAS), while serum ACh and urinary PBG levels were assessed using ELISA and modified Mauzerall-Granick methods, respectively. Results: Mean BLL did not differ significantly between groups, though concerningly high BLLs (up to 112 µg/dL) in some participants indicated significant exposure. Urinary PBG levels were significantly higher in cases (4.03 ± 0.57 µmol/mmol creatinine) compared to negative controls (3.29 ± 0.46 µmol/mmol creatinine). Serum ACh levels were significantly lower in positive controls (439.10 ± 260.69 pg/mL) compared to negative controls (843.19 ± 339.63 pg/mL) but not significantly lower in cases (588.55 ± 239.09 pg/mL). No significant correlation was found between BLL, PBG, and ACh. Conclusion: The study findings are consistent with the potential metal-induced metabolic perturbation in ASD etiology and the role of altered cholinergic neurotransmission in NDD development.

Keywords: Autism spectrum disorder, neurodevelopment disorder, heavy metal toxicity, blood lead level, serum acetylcholine, urine porphobilinogen

Introduction

Autism spectrum disorder (ASD) is one of the most common neurodevelopmental disorders (NDDs) affecting children worldwide. Globally, it was estimated that approximately 1 in 127 persons had autism in 2021 (Institute for Health Metrics and Evaluation, 2024). It is characterized by marked deficits in communication and social behavior (Black & Grant, 2014). Several environmental factors have been implicated in the risk of ASD, including maternal infection during pregnancy (Ohkawara et al., 2015) and prenatal or postnatal exposure to toxic metals (Gorini, Muratori, & Morales, 2014). The recent increase in industrialization and subsequent environmental pollution has heightened attention on the role of heavy metals such as lead (Pb) and mercury (Hg) in the pathophysiology of ASD (Gorini et al., 2014). These metals are known to have severe adverse effects on neurodevelopment, particularly in developing fetuses and infants who have weaker immunity and less developed Blood-Brain Barrier (BBB) (Parithathvi et al., 2024). Compelling evidence indicates that heavy metal exposure increases the risk of mental disorders, including ASD (Bjørklund et al., 2018; Jaishankar et al., 2014). In addition to their direct neurotoxic effects, such as disrupting calcium homeostasis and damaging mitochondria, heavy metals like Pb can impair neurodevelopment by altering the epigenetic function of zinc (Reddy & Zawia, 2000).

Several studies have detected high levels of environmental toxicants, such as Pb and Hg, in the blood (Bjørklund et al., 2018), hair (Fido & Al-Saad, 2005) and urine samples (Bjørklund et al., 2018) of ASD patients, suggesting that lead exposure might be a risk factor for ASD development. Consequently, researchers have investigated biomarkers of lead exposure, such as blood lead level (BLL), as a potential indicator of ASD. Although BLL has been used in many studies to assess Pb exposure, with mixed findings (Omotosho et al., 2018; Bjørklund et al., 2018), it primarily reflects recent or ongoing exposure. This is a limitation, as the risk effects of lead are hypothesized to stem from earlier exposures in life, which BLL does not capture effectively (Kern et al., 2014). Lead has a half-life of 1-2 months in blood and soft tissues but can remain stored in bones for years to decades (Oflaherty, 1993). Therefore, researchers are increasingly focusing on pathways sensitive to metal accumulation, such as the heme biosynthetic pathway, to better assess the total body burden of lead (Austin & Shandley, 2008; Geier & Geier, 2006; Woods, 1996; Bjørklund, 2024; Youn et al., 2010).

Pb and Hg have been shown to disrupt the heme synthesis pathway by inhibiting key enzymes, resulting in the accumulation of intermediates such as aminolevulinic acid (ALA), PBG, and porphyrins (Crook, 2013). Numerous studies have documented elevated levels of porphyrins, such as coporprophyrin and protoporphyrin, in blood and urine samples of ASD patients (Austin & Shandley, 2008; Geier & Geier, 2006; Woods, 1996; Bjørklund, 2024; Youn et al., 2010). However, these studies and many others have failed to investigate the level of PBG, a precursor of porphyrin, as a marker of metal toxicity in ASD patients. This oversight may be due to the technical challenges associated with measuring PBG, as it is unstable and can rapidly polymerize, making it a more difficult analyte than its downstream porphyrin metabolites (Aarsand et al., 2024). PBG accumulates in the blood and is measured in urine during porphyria crisis, which also produces psychiatric symptoms similar to ASD. Moreover, research has proposed that PBG interferes with neurotransmission at the neuromuscular junction (Feldman et al., 1971). This underscores the need for assessing urinary PBG in ASD children, which is one of the focus of this study.

The nervous system of fetuses or infants is particularly sensitive to toxic metals due to the underdeveloped BBB, which allows these metals to penetrate and adversely affect normal brain development and functions (Gorini et al., 2014). Neurotransmitters are crucial for the brain's normal functioning, and alterations in their metabolism and availability have been linked to various psychiatric disorders, including depression, ADHD, schizophrenia, and ASD (McDougle et al., 2005; Seethalakshmi, 2017). Lead (Pb), a divalent cation similar to calcium, has been suggested to cause inappropriate neurotransmitter release at rest and to compete with calcium, disrupting the evoked release of neurotransmitters such as ACh (El-Ansary, Bacha, & Al-Ayahdi, 2011; Lidsky & Schneider, 2003).

Acetylcholine (ACh) is the primary neurotransmitter in the cholinergic system, which regulates behaviors relevant to ASD, including awareness, cognitive flexibility, and communication (Avale et al., 2011). Experimental increases in ACh in mouse models have been shown to improve cognitive deficits associated with psychiatric disorders such as ASD (Wang et al., 2015). Additionally, a randomized clinical trial found that the administration of galantamine, an acetylcholinesterase inhibitor and allosteric modulator of the Alpha7 nicotinic acetylcholine receptor (α7*nAChR), significantly alleviated autism-related symptoms such as irritability and social withdrawal in autistic individuals (Ghaleiha et al., 2014). This

experimental and clinical evidence suggests that modulating ACh can mitigate lead-induced dysregulation of the cholinergic system in ASD pathophysiology. Despite its relevance, ACh is rarely measured in serum due to stability concerns and rapid enzymatic degradation, which limits the number of studies evaluating circulating ACh directly (Świt et al., 2023). Given the potential interaction between metal exposure and cholinergic signaling, this study assesses serum ACh alongside BLL and urinary PBG among Nigerian children with ASD to gain insight new insights into neurochemical alterations associated with ASD.

Methods

Participants

This research is a case-control study comprising 47 participants in age range 3 to 12 years, including 16 children with ASD as cases, 16 children with related NDDs as positive controls and 15 neurotypical children as negative controls. The gender proportion in the ASD group indicated a male-to-female ratio of 7:1, reflecting the known prevalence imbalance in ASD (Werling et al., 2016). We included children with other NDDs (comprising of children diagnosed with attention-deficit/hyperactivity disorder [ADHD], intellectual disability, conduct disorders, and cerebral palsy) alongside children with ASD and typically developing children to explore whether the observed changes in the studied parameters are specific to ASD or common across a spectrum of NDDs. Both cases and positive controls were recruited from the Child and Adult Psychiatry clinic of University College Hospital (UCH), Ibadan, following their diagnosis using DSM-V by a child neurologist and child psychiatrist. Neurotypical children were randomly selected from primary schools in Ibadan. Inclusion criteria includes newly diagnosed subjects (cases and positive controls) in the age range 3-12 years, free of medication and other known diseases such as anemia, diabetes, inflammatory diseases and infection.

Sample Size determination

Based on the data obtained from the past study (Omotosho, Akinade, & Lagunju, 2018), the mean ± SD for BLL in autistic children and the control group were 7.92 ± 1.3 mg/dl and 6.83 ± 0.72 mg/dl, respectively. The sample size was then determined using the following sample size formula for comparing two means (Rosner, 2015):

$$N = (Z_{\frac{\sigma}{2}} + Z_{\beta})^2 \times 2 \times \frac{\sigma^2}{d^2}$$

Where:

$Z_{\alpha/2}$ is the critical value of the normal distribution at $\alpha/2$ (1.96 for 95% confidence), Z_{β} is the critical value of the normal distribution at β (0.84 for 80% power), σ^2 is the population variance (calculated as 1.051), and d is the difference to detect between the means of the two groups (calculated as 1.09). Substituting these values into the formula, N = 14.57. Thus, a minimum of 15 participants was required for each of the two primary comparison groups (ASD vs. neurotypical controls). This sample size was maintained for the three-group design to ensure adequate power for the planned pairwise comparisons.

Ethical considerations

Approval for this study was obtained from the UI/UCH joint Ethical Committee as well as the Oyo State Ministry of Health Ethical Board. Informed written assent and consent were taken from all subjects and their parents, respectively, before specimen collection.

Sample Collection, Processing and Storage

About 5 mL of venous blood was drawn from participants at the time of recruitment into special metal-free plain tubes. Samples were allowed to clot and then centrifuged at 3000rpm for 10 minutes. The serum was separated and stored at -20 °C until assayed. Subsequently, 10 mL of mid-stream urine was collected from the participants into aluminum foil-wrapped urine bottles for quantitative PBG assay; the remaining samples were stored at -20 °C.

Sample Analysis

All biochemical analyses were performed at the postgraduate research laboratory, Department of Chemical Pathology, UCH

Lead Analysis Using AAS

a. Sample Dissolution (Wet Oxidation of the Sample)

Known volume of each sample (between 0.5-3.5 mL) was taken into the digestion flask. 0.5 mL of nitric acid was added to the sample and the mixture was allowed to stay overnight at room temperature. The samples were kept in a drying oven at 60^0c. After cooling, 0.2 mL of 60% H_2O_2 was added, and the samples were incubated for 1hour in the drying oven at 60^0c, allowed to cool, and the digest was made up to 10 mL volume with distilled water.

b. Lead Analysis on AAS

Lead was analyzed using Model 210 VGP of the Buck Scientific AAS series with air-acetylene gas mixture as oxidant. Solutions from the digestion process were aspirated after the equipment was calibrated for the element. The results were recorded in µg/dL. We established a calibration range of 1.0-50 µg/dL for Pd. The limit of detection (LOD) for the method was 0.5 µg/dL.

Serum Acetylcholine Estimation using ELISA

Serum acetylcholine levels were analysed by an Enzyme-Linked Immunosorbent Assay (ELISA) kit specified for biological fluids such as serum (Elabscience Biotechnology Co., LTD) according to the manufacturer's instructions. This method is a modification of the original method described by Engvall and Perlmann (1971)

Quantitative Determination of Urinary Porphobilinogen

Quantitative analysis of PBG in urine was performed using ClinEasy® Photometric Complete Kits (Recipe GmbH, Munich, Germany), which is based on the modified method of Mauzerall and Granick (1956). As PBG and related porphyrin precursors are light-sensitive and to ensure analyte stability, urine samples were protected from light during collection and analyzed immediately.

Urine Creatinine Estimation

Urine creatinine was estimated based on Jaffe method using Architect C4000 (Abbott) autoanalyzer. The Jaffe creatinine method relies on the use of alkaline picrate. When the sample is in an alkaline pH environment, creatinine reacts with picrate to create a complex known as creatinine-picrate. The concentration of creatinine in the sample can be determined by measuring the rate of absorbance increase at 520 nm, which directly correlates with the formation of this complex (Toora & Rajagopal, 2002).

Statistical Analysis

Using SPSS version 25, appropriate statistical analysis was undertaken. Specifically, the normal distribution of the data was assessed using Kolmogorov-Smirnov and Shapiro-Wilk tests. ANOVA was used to compare the means among three groups and a post-hoc test (Tukey's HSD Test) was used to identify the significant pair(s). Kruskal Wallis test was used for data that violate the assumption of one-way ANOVA. Differences between groups at $p < 0.05$ were regarded as significant. Spearman's rank correlation was used to establish the relationship between the studied variables.

Results

The descriptive statistics of the anthropometric (age, weight, height, and BMI) and biochemical variables (BLL, PBG, and ACh) of the 47 study participants are summarized in Table 3.1. The age range for all participants was 3-12 years, with mean ± SD values of 5.13 ± 2.3 years for cases, 7.25 ± 3.3 years for positive controls, and 7.0 ± 2.8 years for negative controls. Weight (kg), height (cm), and BMI (kg/m²) were highest in the negative controls (22.73 ± 4.27, 124.00 ± 10.29, and 16.32 ± 0.76), followed by positive controls (22.32 ± 13.34, 119.43 ± 26.86, and 14.72 ± 2.75), and then cases (19.62 ± 6.30, 109 ± 18.09, and 14.99 ± 1.30), respectively. However, an F-test (Table 3.2) revealed no statistically significant differences in the average age ($p = 0.411$), height ($p = 0.752$), weight ($p = 0.313$), and BMI ($p = 0.150$) across the three groups.

As noted in Table 3.1, BLL values are reported as median ± interquartile range (IQR) rather than mean ±SD due to the highly skewed distribution and the large number of non-detectable readings. More than half of the participants in each group had BLL values below the analytical detection limit (LOQ: 1.0 µg/dL), resulting in a median BLL of 0 µg/dL for cases, positive controls, and negative controls.

A small number of samples, however, exhibited extremely high BLL values, with maximums of 112 µg/dL in cases, 59 µg/dL in positive controls, and 67 µg/dL in negative controls. The presence of these isolated high readings, especially the 112 µg/dL in a child with ASD, is clinically alarming and suggests severe, acute environmental lead exposure in a subset of the study population, irrespective of ASD diagnosis. This high environmental exposure is consistent with the need to investigate associated biomarkers.

Given the non-normal distribution and extreme outliers, a Kruskal-Wallis test was used to compare BLL between groups. The test showed no statistically significant difference in BLL among cases, positive controls, and negative controls ($H = 0.176$, $p = 0.916$), indicating that while severe exposure exists, the sporadic high values did not differ systematically across the three diagnostic groups.

Regarding urinary PBG, cases had the highest mean of 4.03 µmol/mmol creatinine, with minimum and

maximum values of 3.01 and 4.74 µmol/mmol creatinine, respectively. Positive controls had a mean of 3.69 µmol/mmol creatinine, while negative controls had a mean of 3.29 µmol/mmol creatinine. In terms of serum ACh, both cases (262.72-1062.49 pg/mL) and positive controls (60.05-1037.83 pg/mL) had lower ranges compared to negative controls (476.12-1621.63 pg/mL).

Table 3.1 Descriptive Statistics of Studied Variables Among Cases, Positive Controls and Negative Controls

Variables	Cases (n=16)				Positive controls (n=16)				Negative controls (n=15)			
	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
Age	3	9	5.13	2.23	2	12	7.25	4.33	3	13	7.00	3.34
Weight (kg)	13	29	19.63	6.30	13	53	22.31	13.35	15.70	30	22.74	4.27
Height (cm)	90	135	109.0	18.09	90	173	119.44	26.86	105.50	137.50	124.0	10.29
BMI (kg/m^2)	15.40	17.73	16.20	0.77	10.25	18.49	17.02	2.75	13.26	16.00	14.61	0.98
BLL (µg/dL)	0.00	112.00	0.00[a]	45.00[b]	0.00	59.00	0.00[a]	13.00[b]	0.00	67.00	0.00[a]	46.00[b]
Urine PBG (µmol/mmol creatinine)	3.01	4.74	4.03	0.57	2.72	4.51	3.69	0.45	2.43	4.26	3.29	0.46
Serum ACh (pg/mL)	262.76	1062.49	588.55	239.09	60.05	1037.84	439.10	260.69	476.12	1621.63	843.19	339.63

BMI = body mass index, BLL = blood lead level, Pb = blood level, PBG = urine porphobilinogen, ACh = serum acetylcholine.

[a] Measure of central tendency based on Median
[b] Measure of dispersion based on interquartile range (IQR)

A comparison of age and anthropometric measures among the three groups was conducted using an F-test. The results, summarized in Table 3.2, revealed no statistically significant differences in average age (p = 0.411), height (p = 0.752), weight (p = 0.313), and BMI (p = 0.150) across the groups. This indicates that the study participants were age-matched and showed no significant variation in their anthropometric parameters.

Table 3.2. Comparison of Age, Weight, Height and BMI Between Cases and Controls Using ANOVA

Variables	Cases	Positive controls	Negative controls	F-test	p-value
Age	5.13 ± 2.23	7.25 ± 4.33	7.00 ± 3.33	0.924	0.411
Weight (kg)	19.62 ± 6.30	22.32 ± 13.34	22.73 ± 4.27	0.289	0.752
Height (cm)	109 ± 18.09	119.43 ± 26.86	124.00 ± 10.29	1.229	0.313
BMI (kg/m^2)	16.32 ± 0.76	14.72 ± 2.75	14.99 ± 1.30	2.078	0.150

The values of age, weight, height and BMI were expressed as mean ± standard deviation.

To examine the presence of metal toxicity in the pathophysiology of ASD, BLL were analyzed and compared between cases and controls. Although the mean values reported in Table 3.3 indicated higher BLL among cases compared to controls (positive and negative), the comparison was made using median values. An independent sample Kruskal-Wallis H test revealed no significant difference in BLL between the cases and controls.

To further investigate the effect of toxic metals, such as lead (Pb), in ASD, the quantitative urinary level of PBG in cases and controls was analyzed. The results, shown in Table 3.3, demonstrated a significant difference in the mean urinary PBG between cases and controls.

Table 3.3. Comparison of BLL, Serum ACh and Urine PBG Between Cases and Controls

Variables	Cases	Positive Controls	Negative Controls	F-test	P-value
BLL (µg/dL)	0.00±45.00[a]	0.00±13.00[a]	0.00±46.00[a]	0.176[b]	0.916
Urine PBG (µmol/mmol creatinine)	4.03±0.57	3.69±0.45	3.29±0.46	7.261	0.002*
Serum ACh (pg/mL)	588.55±239.09	439.10±260.69	843.19±339.63	8.332	0.001*

*The mean difference is significant at the 0.05 level

[a]Median±IQR
[b] Independent sample Krukal Wallis H test score

A post-hoc test (Tukey's HSD) results, summarized in Table 3.4, further revealed that mean urine PBG was significantly higher in cases than in negative controls (p = 0.001, 95% C.I. = [0.26, 1.20]), while the mean differences between cases and positive controls (p = 0.205) as well as between negative and positive controls (p=0.118) were not statistically significant.

Table 3.4. Tukey HSD Post-Hoc Test Results for Urinary PBG

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cases	Positive controls	0.33477	0.19257	0.205	-0.1354	0.8049
	Negative controls	0.73354*	0.19257	0.001	0.2634	1.2037
Positive controls	Cases	-0.33477	0.19257	0.205	-0.8049	0.1354
	Negative controls	0.39877	0.19610	0.118	-0.0800	0.8776
Negative controls	Cases	-0.73354*	0.19257	0.001	-1.2037	-0.2634
	Positive controls	-0.39877	0.19610	0.118	-0.8776	0.0800

*. The mean difference is significant at the 0.05 level.

To determine the possible involvement of the cholinergic system in ASD development, serum levels of acetylcholine were estimated and compared between cases and controls. The results, presented in Table 3.3, showed a significant difference in serum acetylcholine levels among the three groups. Specifically, Tukey's HSD test for multiple comparisons (Table 3.5) found that the mean serum acetylcholine level was significantly

different between cases and negative controls (p = 0.037, 95% CI = [12.51, 496.98]) as well as between positive and negative controls (p = 0.001, 95% CI = [161.86, 646.33]). However, there was no statistically significant difference between cases and positive controls (p = 0.291).

Table 3.5. Tukey HSD Post-Hoc Test Results for Serum ACh

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Cases	Negative controls	149.34875	98.24662	0.291	-88.9467	387.6442
	Positive controls	-254.74792*	99.87064	0.037	-496.9824	-12.5135
Negative controls	Cases	-149.34875	98.24662	0.291	-387.6442	88.9467
	Positive controls	-404.09667*	99.87064	0.001	-646.3311	-161.8622
Positive controls	Cases	254.74792*	99.87064	0.037	12.5135	496.9824
	Negative controls	404.09667*	99.87064	0.001	161.8622	646.3311

*. The mean difference is significant at the 0.05 level.

To gain insight into the possible association between the toxic metal lead (Pb), its indirect measure porphobilinogen (PBG), and the cholinergic neurotransmitter acetylcholine (ACh), a Spearman's rank-order correlation was performed among the biochemical parameters. The results, as shown in Table 3.6, revealed no significant relationships between any of the variables across the three groups.

Table 3.6. Correlations Between Biochemical Variables Among Cases, Positive Controls and Negative Controls

Variables	Cases		Positive controls		Negative controls	
	ρ	p-value	ρ	p-value	ρ	p-value
Pb/PBG	-0.448	0.108	0.094	0.760	-0.478	0.098
Pb/ACh	0.242	0.404	-0.235	0.440	-0.141	0.646
PBG/ACh	0.210	0.472	-0.418	0.156	0.406	0.169

'ρ' indicates Spearman's rank coefficient. Pb = blood lead level, PBG = urine porphobilinogen, ACh = serum acetylcholine.

Discussion

NDDs are groups of disorder that affect the brain functions, thereby altering social, cognitive, and emotional functioning of affected individuals. One of the most common NDDs is ASD. The need for their proper and effective diagnosis is informed by the consequences of the disorder, which in later years result in clinical abnormalities of children with the disorders. This study attempts to support global empirical inquiry into role metal toxicity and cholinergic neurotransmitter in the aetiogenesis of ASD by investigating blood lead level, urinary PBG and serum ACh in Nigerian children with ASD. To achieve this objectives, 16 ASD children diagnosed by a child psychiatrist using DSM-5 were recruited from the Department of Child and Adult Psychiatry, UCH, Ibadan. The age and anthropometric characteristics (weight, height and BMI) of cases were not significantly different from those of controls. This use of use of age- and anthropometrically-matched controls, including a group with other NDDs, in this study enables reasonable comparison of biochemical parameters between cases and controls with close biological makeup, which is essential to limit the effect of cofounding and ultimately increase the validity of the results (Rose & Laan, 2009). The inclusion of this broad NDD group was critical to investigate whether the observed biochemical alterations are specific to ASD or represent a shared feature across a spectrum of neurodevelopmental conditions, thereby offering insights into the specificity of the proposed pathophysiological mechanisms.

Pre- and post-natal exposure to heavy metals has been implicated in the pathophysiology of ASD, consistent with the growing industrial use and emission of heavy metals into the environment over the past decades (Gorini et al., 2014). Lead (Pb) is a non-essential toxic and heavy metal and can be found extensively in the environment (Bjørklund et al., 2018). Several research has established a link between lead exposure and ASD. Eppright et al. (1996) conducted one of the initial studies on this topic, revealing a notably high BLL of 42 µg/dL in a child diagnosed with both ASD and ADHD. These findings suggest the potential relevance of lead toxicity markers in ASD diagnosis. In this study, the median BLL was zero across all three groups, with more than half of the participants having levels below the detection limit. This suggests that BLL may not adequately capture the long-term, low-level exposure hypothesized to be relevant to ASD etiology. However, BLL maxima observed across the cohort, including an alarming 112 µg/dL in the ASD group and 67 µg/dL in neurotypical controls, indicate that severe, acute environmental lead exposure affects a critical subset of this Nigerian population, making the investigation of lead biomarkers highly relevant (Akinwunmi et al., 2023). A rank-based nonparametric test revealed no significant difference in BLL between the cases and controls. This is consistent with the study by Akinade et al. (2019) and Omotosho et al. (2018) in Nigeria, which found that autistic children had higher BLL than neurotypical children, although the difference was not statistically significant. The indifference in BLL between both neurodiverse and neurotypical children, as underscored in this study, may indicate that BLL alone may not explain the contribution of lead exposure to the pathophysiology of NDDs, particularly ASD.

Although traditional analysis of heavy metals of lead in bodily fluids and tissues has been useful in establishing the increased level of heavy metal exposure in ASD patients compared to neurotypical individuals, it only reflects the level of current exposure and not on the body metal burden (Kern et al., 2014). Meanwhile, ASD is hypothesized to be an outcome of chronic exposure to heavy metals, particularly from the pre-natal to early post-natal periods (Bjørklund et al., 2018). This underscores our analysis of biomarker of effect of Pb exposure in explaining the role of metal toxicity in ASD aetiology. Moderate exposures to Pb can cause overall toxicity in the heme synthetic pathway by stimulating the activity of delta-ALA synthetase (delta-ALA-S), the mitochondrial enzyme responsible for the rate-limiting step in heme formation, as well as inhibiting the activities of the cytosolic enzyme porphobilinogen synthetase (PBG-S or delta-ALA-D) and the mitochondrial enzyme ferrochelatase. The effects include the accumulation of porphyrins and its precursors (ALA and PBG) in tissues and urine, which can be quantified as an indicator of lead toxicity (Crook, 2013). Accordingly, our study indicated a statistically significant higher level of urine PBG in ASD children than in neurotypical children. Similarly, children with NDDs other than ASD had higher mean urine PBG compared to neurotypical children; however, the difference is not statistically significant. We acknowledge that elevated urinary PBG can be non-specific, potentially caused by factors such as acute hepatic dysfunction, certain medications, or severe hemolysis (Crook, 2013); however, these co-morbidities were ruled out by our inclusion criteria.

The higher urinary PBG observed in autistic children compared to neurotypical children complements past studies, which found an increase in porphyrin excretion among ASD patients (Nataf et al., 2006; Woods et al., 2010; Youn et al., 2010). Notably, the study conducted by Geier and Geier (2006) revealed a dose-response relationship between increased urinary coproporphyrin levels and the severity of autism. This finding suggests a potential causal effect of these metabolites on brain chemistry, albeit with unclear mechanism. The association could explain the psychiatric symptoms observed in patients with acute porphyria, often characterized by the accumulation of PBG in blood and ultimately positive urinary PBG (Ricci et al., 2021). While raised urine PBG demonstrated a positive correlation with lead toxicity (Gibson, Mackenzie, Goldberg, & Medicine, 1968), the lack of significant correlation between urine PBG and BLL in this study may suggest other toxic metals like mercury (Hg) are in play. Like Pb, Hg exposure results in the perturbation of heme synthetic pathway, resulting in the accumulation of intermediates such as PBG (Schauder et al., 2010). Besides, the raised PBG in cases compared to controls could suggest chronic exposure to Pb rather than recent exposure, which may not be accurately captured by BLL (Bjørklund et al., 2018).

Neurotransmitters play an important role in the mental and physical behaviour by facilitating communication between nerve cells. Therefore, their increased and decreased synthesis could explain some of the behavioural and communication deficits associated with ASD. Acetylcholine is one of the major primary neurotransmitters in the autonomic nervous system, allowing for the activation of muscles (Karvat & Kimchi, 2014). It also supports cognitive functions such as learning, memory and attention in the central nervous system (Seethalakshmi, 2017). Increased or decreased production of ACh may result in mental illnesses, such as depression, Alzheimer's disease and Parkinson's disease. Studies on ACh in ASD have been limited to examining the abnormalities in the cholinergic system at the receptor level (Marotta et al., 2020). It is worth noting that measuring ACh in a peripheral fluid like serum presents inherent complexity and limitations, as these levels may not directly reflect ACh activity in the central nervous system (CNS) due to the blood-brain barrier, and thus serve as an indirect, peripheral marker (Nwobi et al., 2019). Acetylcholine in serum is reported to exert an effect on the muscarinic receptor present on the vascular endothelium, thereby promoting vasodilation (Kawashima et al., 1997). This study assessed the level of serum ACh in ASD cases and neurotypical children. The comparison revealed no significant difference in serum ACh between ASD cases and neurotypical children. However, the children with NDDs other than ASD had a statistically significant lower serum ACh than the neurotypical children. Furthermore, ACh levels were significantly higher in cases than in positive controls.

The finding that ACh levels were significantly depressed in the positive controls compared to the neurotypical children, but not significantly depressed in ASD cases compared to neurotypical children, highlights that altered peripheral ACh is not unique to ASD. Instead, it may reflect a common dysfunction in the cholinergic system spanning various NDDs or may be highly susceptible to environmental factors. Nwobi et al. (2019) reported an increase in acetylcholinesterase activity induced by Pb exposure, which leads to the hydrolysis of ACh. Similarly, a study by Feldman et al. (1971) demonstrated that increased PBG, which can be potentiated by Pb toxicity, can inhibit the stimulation of motor nerve terminals following depolarization with potassium ions, ultimately reducing the release of the

neurotransmitter ACh during a nerve action potential. Therefore, exposure to Pb and the resulting metabolic effect may influence the cholinergic system across NDDs. However, since this study failed to establish a significant association neither between ACh and BLL nor between ACh and PBG, the observed serum ACh pattern is open to several possible interpretations: First, decreased synthesis due to nutritional factors such as inadequate dietary choline status, a precursor of ACh. This interpretation is consistent with previous studies, which found a decreased concentration of choline in brain tissue (Friedman et al., 2006) and plasma (Hamlin et al., 2013) of ASD subjects. Second, divalent heavy metals other than Pb could be involved. For example, Hg could interfere with calcium ion-induced ACh release. This is corroborated by the finding of a reduced plasma calcium level in ASD children compared to neurotypical children (Omotosho et al., 2018). Third, ACh is unstable and can be rapidly hydrolyzed by Acetylcholinesterase following sample collection and storage, despite precaution, resulting in reduced ACh detection (Swit, 2023).

The lower ACh concentration observed in children with NDDs other than ASD may also highlight the underlining role of the cholinergic system in the development of NDDs. In particular, the cholinergic system regulates behaviours relevant to NDDs, including cognitive flexibility (Ragozzino et al., 1998), awareness (Arnold et al., 2002) and communication (Avale et al., 2011). This is supported by the study of Karvat and Kimchi (2014), which found that increasing ACh in the synaptic cleft by inhibiting acetylcholinesterase reduces autistic phenotypes in a mouse model. Furthermore, a study using a mouse model revealed that augmenting the levels of ACh could ameliorate the cognitive deficits associated with psychiatric disorders, such as ASD (Wang et al., 2015). Similarly, a randomized clinical trial demonstrated that the administration of Galantamine, an acetylcholinesterase inhibitor and allosteric modulator of α7*nAChR, significantly alleviated autism-related symptoms such as irritability and social withdrawal in individuals with autism (Ghaleiha et al., 2014). In line with these findings, the observed disparity in the serum level of ACh between positive controls and neurotypical children may implicate dysfunctional cholinergic system in the development of NDDs.

Conclusion

The prevalence of ASD is rising globally, yet its underlying etiology remains elusive. Numerous studies have suggested that heavy metal toxicity and altered neurochemistry play roles in the pathophysiology of ASD. This research contributes to the expanding body of literature on ASD, particularly within the African context where such investigations are limited, by assessing BLL, serum ACh and urinary PBG in ASD children and age-matched controls. Notably, the study found no significant difference in BLL between the cases and controls, suggesting a lack of systematic, high-level recent Pb exposure among the majority of the study participants. However, the presence of isolated, critically high BLL outliers necessitates continued vigilance regarding acute environmental exposure. The elevated urinary PBG observed among the cases compared to controls is consistent with the notion of metabolic perturbation potentially linked to chronic heavy metal exposure (Pb and/or Hg). Furthermore, the study did not find a significant difference in serum ACh between ASD cases and neurotypical controls. However, the significantly lower serum ACh found in the children with NDDs other than ASD (positive controls), compared to the neurotypical children, underscores the possible role of a dysfunctional cholinergic system in the development of NDDs more broadly. While several studies have linked BLL to altered neurotransmission, this study failed to establish a significant relationship between BLL, PBG and ACh. Overall, the study contributes to the existing body of knowledge on the role of environment toxicity and altered neurochemistry in ASD etiology.

Limitations

Despite the tangible contributions of this study, it is not bereft of limitations, which could be exploited for future studies. First, the study's sample size is small, which could reduce the strength of our observation. Second, the case subjects were not sex-matched with the controls, which may have reduced the statistical efficiency and introduced potential confounding, given the sex differences in NDDs. Third, the study's population is limited to participants from Ibadan, Nigeria. Thus, caution may be necessary in generalizing the findings. Fourth, due to limited resources, several important parameters were excluded in the analysis, including blood mercury and urine aminolevulinic acid (ALA). Including these analyses could enrich the understanding of the influence of heavy metals on the heme pathway among the three groups. Fifth, AAS was used for the metal (Pb) analysis. This method is less sensitive compared to more advanced techniques like Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Sixth, the analysis of serum ACh is subject to limitations due to its rapid degradation and potential instability during sample processing and storage. Lastly, the elevated urinary PBG cannot be definitively attributed to metal toxicity, as alternative pathways (such as liver dysfunction or drug use) that were

controlled by inclusion criteria were not specifically ruled out by biochemical markers.

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Statements and Declarations

Ethics approval and consent to participate: This study was performed in line with the principles of the Declaration of Helsinki. Approval for this study was obtained from the UI/UCH joint Ethical Committee as well as the Oyo State Ministry of Health Ethical Board. Informed written assent and consent were taken from all subjects and their parents, respectively, before specimen collection.

Consent for publication: Not applicable.

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