TRACE METALS, NEUROTRANSMITTERS AND OXIDATIVE STRESS MARKERS IN THE PATHOGENESIS OF AUTISM SPECTRUM DISORDERS AND CEREBRAL PALSY IN CHILDREN

BY

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CERTIFICATION

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DEDICATION

This thesis is dedicated to Jesunifemi, my caring and loving son, whose childhood challenge brought me to the field of Neurodevelopmental Disorders and Neurotoxicology.

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ABSTRACT

Autism Spectrum Disorders (ASD) and Cerebral Palsy (CP) are Neurodevelopmental Disorders (NDDs) with inconclusive genetic profiling. Currently, focus is on gene modulation in NDDs by environmental toxicants such as trace metals which induces oxidative stress. However, interrelationship between oxidative stress and neurotransmitters in the pathogenesis of NDDs is unclear. This study was conducted to assess *in-utero* placenta transfer of trace metals from occupationally-exposed pregnant mothers and effect on neurotransmitters in the pathophysiology of ASD and CP in children.

Ethical approvals were obtained from UI/UCH Ethics Committee (UI/EC/15/0087) and Oyo State Ministry of Health. This case-control study had 180 participants; 50 pregnant women occupationally-exposed to metals (cases), 55 unexposed (controls), 25 each clinically-diagnosed ASD, CP and Neuro-typical (NT) children, respectively. Maternal and cord blood obtained at parturition and blood samples from ASD, CP and NT were analysed for trace metals (selenium, zinc, copper, calcium, magnesium), lead, cadmium using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Samples from ASD, CP and NT were also assayed for neurotransmitters (glutamine, glutamate, GABA) using ELISA. Malondialdehyde (MDA), Total Antioxidant Capacity (TAC) and Total Plasma Peroxide (TPP) were spectrophotometrically determined, while Oxidative Stress Index (OSI) was calculated (TPP/TAC). Data were analysed using ANOVA and Pearsons' correlation at $\alpha_{0.05}$.

Maternal selenium, zinc, copper, lead, cadmium, calcium, magnesium levels in exposed (10.2±1.2, 370.8±193.0, 328.0±110.0, 11.0±1.4, 96.7±15.6 µg/dL; 8.6±0.9, 1.5±0.3 mg/dL) and unexposed (9.0±1.2, 416.8±277.0, 348.3±150.6, 10.0±1.9, 70.0±30.0 µg/dL; 8.6±0.9, 1.5±0.4 mg/dL) pregnant women were not significantly different. In the fetus cord-blood, selenium, zinc, copper and calcium levels were not significantly different, magnesium level (1.51±0.3 vs 1.6±0.1) was significantly reduced in exposed compared with unexposed, while lead and cadmium were not detectable. In ASD and CP, compared with NT, plasma calcium (7.9±1.4; 7.7±1.0 vs 9.8±1.3 mg/dL), magnesium (2.5±0.5; 2.8±0.6 vs 3.1±0.4 mg/dL), selenium (40.8±7.9; 27.6±6.8 vs 59.0±5.3 µg/dL), zinc (222.3±63.8; 233.8±105.3 vs 438.5±185.5 µg/dL) and copper (4.3±1.0; 4.0±0.8 vs 4.9±0.9 µg/dL) were significantly reduced, while lead level (9.5±4.0; 11.1±5.8 vs 5.4±2.05 µg/dL) was significantly elevated. The Zn/Cu ratio (55.3±22.0; 60.6±27.8 vs 92.3±44.6) was significantly reduced in ASD and CP compared with NT. Glutamine level (379.2±53.1; 296.3±59.6 vs 419.1±71.8 µmol/L) was decreased significantly in ASD and CP compared with NT. Glutamate (1.9±0.1; 1.8±0.3 vs 1.7±0.3 nmol/mL) and GABA (2.1±0.3; 1.8±0.4 vs 1.8±0.3 µmol/L) levels in CP and NT were comparable, and significantly elevated in ASD compared with NT. The OSI (0.6±0.2 vs 0.4±0.1; 0.4±0.1) and TPP (115.1±8.5 vs 105.9±2.3; 110.4±7.9) levels were significantly higher and TAC (209.8±57.9 vs 280.2±34.4; 303.8±33.1) was significantly reduced in CP compared with ASD and NT. The MDA (2.3±0.2; 2.1±0.2 vs 1.4±0.1) level was significantly elevated in ASD and CP compared with NT. Copper correlated positively with GABA and glutamine, while magnesium correlated negatively with GABA in ASD. Copper correlated positively with glutamate in CP.

Transfer and imbalance of trace metals *in-utero* was established. Oxidative stress observed in autism spectrum disorders and cerebral palsy can be ascribed to imbalance in trace metals resulting in abnormal glutamatergic and GABAergic neuron activities in children with these disorders.

Keywords: Autism spectrum disorders, Cerebral palsy, Neurodevelopmental disorders, Trace metals.

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LIST OF ABBREVIATIONS

(ADDM):	Autism and Developmental Disabilities Monitoring
(ADHD):	Attention-Deficit Hyperactivity Disorder
(ASD):	Autism Spectrum Disorder
(ATSDR):	Agency for Toxic Substances and Disease Registry
(CDC):	The Centres for Disease Control and Prevention
(CP):	Cerebral Palsy
(HPV):	High Production Volume
(ID):	Intellectual Disability
(MR):	Mental Retardation
(ncRNAs):	non-protein-coding RNA
(NDDs):	Neurodevelopmental Disorders
(DSM):	Diagnostic Statistical Manual of Mental Disorders
(SNPS):	Single Nucleotide Polymorphisms
(THMS):	Trihalomethanes
(WHO):	World Health Organization
µl	Microlitre
^0C	Degree Celsius
ADH	Antidiuretic Hormone
ALA	δ-Aminolevulinic Acid
As	Arsenic
ATOX1:	Antioxidant Protein 1
BBB	Blood Brain Barrier
Ca	Calcium
Cd	Cadmium
Cm	Centimeter
CNS	Central Nervous System
Cu	Copper
EPA	Environmental Protection Agency
GIT	Gastrointestinal Tract
ICD	International Classification of Disease
Kg	Kilogramme
Kg/m^2	Kilogram per metre square

M	Metre
Mg	Magnesium
Mg/dl	Milligram per deciliter
Ml	Millilitre
Mn	Manganese
NAS	National Academy of Sciences
NCV	Nerve Conduction Velocity
NCV	Nerve Conduction Velocity
Nm	Namometre
Pb	Lead
PCBs	Polychlorinated Biphenyls
Pmol/l	Picomole per litre
Ppb	parts per billon
PTH	Parathyroid Hormone
PTH	Parathyroid Hormone
Se	Selenium
TALH:	Thick Ascending Loop of Helen
U/L	International unit per litre
V	Vanadium
VDR	Vitamin D Receptor
Yrs	Years
Zn	Zinc
%	Percent

PUBLISHED PAPERS

Omotosho, I. O., **Akinade, A. O.**, & Lagunju, I. A. 2018. Calcium and Magnesium Levels Are Down Regulated in Nigerian Children with Autism Spectrum Disorder and Cerebral Palsy. *Neuroscience & Medicine*, 9: 159-170.

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CHAPTER ONE

INTRODUCTION

1.1. BACKGROUND TO THE STUDY

Neurodevelopmental disorders (NDDs) are disabilities primarily caused by impairment of the neurological system and brain functioning (ACE, 2015). As a consequence of abnormal brain growth in the disorders, they are marked by deficit in memory, communication, actions as well as motor abilities (Leonard, 2016). Examples of NDDs are Cerebral palsy (CP), Autism Spectrum Disorder (ASD), Dyslexia, Attention-deficit hyperactivity disorder (ADHD) and other cognitive impairments; throughout the world, these disorders affect millions of children (WHO, 2012). About 10–15% of all births suffered from these disorders of neurobehavioral development (Dubovický, 2010).

Mental retardation, cerebral palsy, and autism spectrum disorders are the three commonest chronic developmental disorders that cause suffering in the affected children as well as their families. The recent estimated prevalence of neurodevelopmental disabilities and ASD in particular among American children between ages 3 to 17 years alone were 1 in 28 (3.57%) and 1 in 45 (2.24%) respectively (Nguyen Quoc & Kunio Miyake, 2017).

Autism spectrum disorders (ASDs) are a broad term that refers to a variety of neurological conditions which are marked by deficit in verbal, nonverbal communication, cognitive, repetitive habits, stereotype behaviour and interests starting during infancy and toddler years. The distinctive social behaviours include problems associated with emotional regulation and compassion for others' feelings, an avoidance of eye-to-eye contact and a significantly limited spectrum of interests and behaviors (WHO, 2012; APA 2013; Wu *et al.,* 2015; Bakroon & Lakshminarayanan 2016; WHO,2018; Hoang *et al.,* 2019).

ASDs mainly include social communication and behavioural disorders according to DSM-5 classification (APA, 2013; McPartland *et al*., 2016). Subclinical reduction in function of the brain is more common than developmental neuro-behavioural issues. The consequences of these disabilities can be serious on the developmental processes of the body and these include diminished quality of life, low academic performance, and unpredictable behaviour, all of which have significant consequences for general societal welfare and productivity (Gould, 2009). Several factors have been identified in the ASD pathophysiology, but researchers hypothesized that environmental toxicants, or other contaminants contribute to the development of these disorders (Grandjean, 2013). This is also supported by a child's developing brain weakness *in-utero* (Grandjean & Landrigan 2014). Others have suggested strong links between genetic modulation by these toxicants and the development of ASD, emphasizing the pathophysiology of ASD complexity. Many researchers believe that genetic and environmental influences, as well as their interactions contribute significantly to the development and progression of these disorders (Chaste & Leboyer, 2012; Esposito *et al*., 2018; Rylaarsdam & Guemez-Gamboa, 2019). The increasing incidence of these disorders around the world has increased the likelihood of this claim.

The prevalence rates of autism spectrum disorders seems increasingly high globally and it affects boys 4 times more frequently than girls. ASD is globally reported to have prevalence of 7.6 cases per 100 (1 in 132) (Baxter *et al.,* 2015). According to Centres for Disease Control and Prevention (CDC), 1.13% (1 of 88), 1.47% (1 in 68), 1.69% (1 in 59), 1.86% (1 in 54) and 2.22% (1 in 45) of American children were affected by ASD in 2008, 2012, 2014, 2016 and 2018 respectively (CDC 2012; CDC 2014; CDC, 2015; CDC, 2018). In Nigeria, the prevalence also appears to be on the increase, rising from 0.8% in 2011 (Bakare *et al*., 2011a) to 2.3% in 2014 (Lagunju *et al*., 2014) according to hospital-based evidence.

The origins of the current global neurodevelopmental disorder pandemic are only partially known. Other genetic syndromes, like Fragile X and Rett syndrome linked to ASD could not explain the recent record rise in the incidence of ASD, as the discovered genes are only responsible for a small percentage of the cases (NRC. 2000). Based on empirical research, single gene and genetical abnormalities accounted for just a small incidence of ASD cases (Schaefer & Mendelsohn, 2013; Lyall *et al*., 2017). According to new evidence, environmental factors have recently been linked to ASD.

Environmental factors were projected to account for about 55 percent risk of developing autistic disorders, while genetic factors were responsible for about 37 percent according to study of 192 twin-pairs (Hallmayer *et al*., 2011).

While several psychological and behavioural ASD characteristics are thought to be triggered by CNS dysfunction, evidence from a range of fields of medicine has related ASD to certain physiological abnormalities in other systems other than the nervous organs, indicating that ASD could be caused by anomalies that affect the whole body rather than just one organ in some people (Rossignol & Frye, 2012; Ha *et al*., 2015). Researches in the recent have linked detoxification impairments, imbalance redox activities, immune dysregulation, inflammation, and energy generation to metabolic systems dysfunction rather than particular organ malfunction (Ming *et al.,* 2008; Rossignol & Frye, 2012). This indicates that ASD could be caused by systemic functional anomalies rather than a strictly brain and nervous system conditions (Hodges *et al*., 2020).

The role of essential and toxic elements (unavoidable contaminants) in gene growth in subjects who are directly or indirectly exposed to toxicants becomes critical in this regard. Environmental factors were long related to a range of neurodevelopmental deficits. There is a lot of proof that synthetic chemicals commonly dispersed in the environment contribute to this global yet silent pandemic of neurodevelopmental toxicity by damaging the developing brain. (Grandjean, 2013; Behl *et al*., 2020). Environmental exposures to toxins during pregnancy have been linked to the aetiology of cognitive impairments, motor, behavioral and sensory disorders; encephalopathy, convulsions or paralysis have also been associated with exposure of children prenatally to high doses of heavy metals (Liu and Lewis, 2014).

However, the burden of lead (Pb) as well as other toxic metals is one environmental factor which has received a lot of attention (Blaurock-Busch *et al*., 2011). Toxic material exposures are especially dangerous to the developing human brain, within critical developmental periods *in-utero,* infancy and child development (Grandjean & Landrigan, 2014). Toxic substances can cause irreversible brain damage even at low exposure levels, which would have next to no effect on an adult during these sensitive stages of life. It then becomes clear that many metals play vital roles in human wellbeing, and that trace elements deficiency could be a major factor in a number of mental health issues. Too much of toxic metal exposure or a deficit of an essential elements can cause

an imbalance (Lakshmi Priya & Geetha, 2010). Aside from the crucial roles those essential elements play in maintaining good health, they also help to reduce the quantity of harmful metals from the environment to the body by chelating them.

Heavy metals are metallic elements and metalloids that occur naturally in the earth's crust with relatively high density when compared to water. They are found in low concentrations and cause toxicity when exposed to them at low levels (Martin Koller & Hosam Saleh, 2018). Toxic metals are minerals that have little or unknown functions in the body. Toxic elements include arsenic (As), cadmium (Cd), lead (Pb) and Mercury (Hg) (Brian, 2012). Mankind is currently exposed to the increased levels of toxicants ever reported. This is due to industrial use of a number of toxic metals and chemicals in the past 300 years without proper waste management and scrubber for combustion of fossil fuels. Toxicants are now present all over the world, harming everyone by impairing health status. They have turned into a major source of diseases, early aging and genetic defects (Singh *et al*., 2011).

When a pregnant woman is exposed to one or more toxic elements, there is concern not just for the mother's health and well-being, but also for the fetus's safety and well-being (Zaw & Taneepanichskul, 2019). They pass through the human placenta and have the potential to harm the fetus during the crucial developmental stages, influencing health later in life (Punshon *et al*., 2016). Exposure to toxic elements during pregnancy has been a serious concern, since it has been established as part of the main causes of prenatal stress (Maekawa *et al*., 2017). Toxic heavy metal exposure causes prenatal stress which is thought to be the cause of many neurological disorders in children, including decreased cognition, attention deficit, and vasomotor difficulties (Maekawa *et al*., 2017).

These environmental toxicants have the potential to cause developmental plasticity and adversely affect endocrine regulation of development in the foetus. When organs are developing in the uterus, foetuses are most vulnerable to environmental toxicants; anomalies at this time cause neuro-behavioural functioning, impaired neurodevelopmental outcomes and low birth weight (Gluckman *et al*., 2008; Carroquino *et al*., 2012). The effects of Pb and Cd on the body, especially in minors, are receiving attention, as environmental toxic metals have no acceptable safe level. Also, increased Pb levels in the circulation during pregnancy can cause low birthweight, delay in development and miscarriage. Cd and pb actively cross placenta, which can trigger fetal

neurodevelopmental issues (Irwinda *et al.*, 2019). Although trace elements are the fundamental components of human body, essential for body structure, fluid equilibrium, protein structure and hormone production, they are also necessary for optimal health and body systems functions. Most enzymes in the body use them as cofactors, catalysts, or inhibitors. All living things require trace elements for proper growth and metabolic functionality. They help human reproduction by promoting proper cell metabolism, good immune function, and healthy reproduction. Calcium (Ca), Zinc (Zn), selenium (Se), magnesium (Mg) and copper (Cu) are all human vital trace elements in the body. Although these essential elements are involved in numerous enzymes and metabolic activities, one trace element deficiency is frequently associated with a range of signs than a single clinical manifestation. Essential elements also play a crucial function in cell differentiation and cell division in the development of fetal cells (Irwinda *et al.*, 2019). During pregnancy, trace elements are strongly connected to fetal growth and development. Human micronutrient levels are influenced by eating habits, lifestyle and exposure to environmental toxins.

Electron transport system and cellular energy production require iron and copper for optimal function. Also, elements like zinc and selenium are needed to help metabolize and eliminate heavy toxic metals; thus, a deficiency of such nutrients can initiate many metabolic abnormalities. However, Some of the necessary minerals like selenium, iron, manganese, hexavalent chromium and others may be extremely toxic. Too much of even the most needed minerals can also become toxic (Wilson, 2016). Excess or deficiency of micronutrients during pregnancy might result in a negative pregnancy outcome and neurological problems in the fetus.

Pregnant women and children are particularly vulnerable to toxicants from chemicals of High Production Volume (HPV) used in thousands of consumer goods. The Centre for Diseases and Control (CDC) studies revealed a higher level of HPV contaminants nearly in everyone in United States, including women who are pregnant (Woodruff *et al*., 2011). Environmental toxicants like As and Pb have all been linked to neurodevelopmental disorders (Gorin *et al*., 2014; Rauh *et al*., 2016). Such exposures could occur with certain occupations like in women selling by the road side, women involved in mechanical mining or those married to men involved in mechanical mining, welding, spray painting, vulcanizing, battery charging, etc. It has been postulated that toxicants may injure the developing human brain directly or through interactions with the genes.

About 30 % of neuro-behavioural disorders are said to be caused by environmental toxicants while about 25 % was reported to be due to interaction between environment and gene (National Research Council, 2000). Epigenetic "tags" have been shown to alter DNA structure and affect gene expression (Kanherkar *et al*., 2014). The mechanism of interaction between such gene and environment may be epigenetic alteration of gene expression by toxicants which cause DNA methylation or changes in non-protein-coding RNA (ncRNA) activity levels. (Grafodatskaya *et al*., 2010). Furthermore, human genetic vulnerability to environmental toxins may affect responses and lead to increased disease vulnerabilities (Faustman *et al.*, 2000). Studies have reported that certain ASD people lack gene for environmental response, which is involved in environmental pollutants detoxifications (Lawler *et al*., 2004) and over 100 of such genes were said to be contributing to the risk factors of developing ASD (Herbert *et al.*, 2006). Environmental response genes are suspected to have single nucleotide polymorphisms (SNPs) that raise vulnerability to harmful effects of environmental toxicants (Hernandez & Blazer, 2006; Hollman *et al*., 2016). The contribution of environmental toxicants to the pathogenesis of neurodevelopmental disorders (ASD and CP) is largely ignored until recently (Lawler, 2008), necessitating a more comprehensive investigation of their involvement in development of ASD, particularly with the disorders observed rising prevalence.

1.2 RATIONALE FOR THE STUDY

Exposure to neurotoxicants has been linked to neurodevelopmental disorders, especially thedramatic rise in the incidence of ASDs over the recent decades. It has been suggested that prenatal and perinatal exposure to environmental contaminants may interfere with the production of neurotransmitters and receptor expression of the developing brain. This could result in changes in the brain that predispose child to ASD and CP during childhood and adolescence. All these disabilities can have severe consequences. Despite the fact that research encompassing genetic and environmental factors has been conducted to determine the causes of neurodevelopmental disorders (NDDs) in recent time, this has not deter an increase in the incidence of ASD in many parts of the world, including Nigeria. Despite this, there has been is no concerted research effort, information and education on these disorders in Nigeria. There are major gaps in knowledge on the effect of environmental toxicants on a child's developing nervous system and the effect of trace elements in relation to the pathogenesis of ASD and CP, especially in this country. This study, therefore, examined possible placental transfer of

some environmental neuro-toxic and essential elements and relate their effect on some neurotransmitters and oxidative stress markers to the pathogenesis of ASD and CP in children. This will generate scientific data that may be used in the diagnosis, management and prevention of these disorders in this environment.

1.3 AIM OF THE STUDY

The aim of the study was:

To investigate possible placental transfer of trace elements in occupationally exposed pregnant women and evaluate levels of trace elements, neurotransmitters and oxidative stress markers in clinically-diagnosed children with ASD and CP.

1.4 STUDY OBJECTIVES

The specific objectives of this study were:

I. Estimate levels of toxic (cadmium and lead) and essential elements (calcium, magnesium, zinc, copper and selenium) in occupationally exposed and unexposed pregnant women and corresponding cord blood.

II. Determine the relationship between levels of toxic (cadmium and lead) and essential elements (calcium, magnesium, zinc, copper and selenium) in maternal and cord blood in occupationally exposed and unexposed groups

III. Determine the relationship between levels of toxic (cadmium and lead) and essential elements (calcium, magnesium, zinc, copper and selenium) in occupationally exposed and unexposed pregnant women with babys' anthropometric indices.

IV. Investigate possible placental transfer of toxic and essential elements *in-utero* through umbilical cord in the exposed pregnant women.

V. Estimate the levels of toxic (Lead, Manganese, Arsenic, Aluminium and Vanadium)and essential elements (Magnesium, Calcium, Copper, Selenium and Zinc) in children with ASD, CP and NT.

VI. Estimate levels of neurotransmitters (Glutamine, glutamate and GABA) in ASD, CP and controls.

VII. Determine the relationship between levels of neurotransmitters (Glutamine, glutamate and GABA) and trace elements in children with ASD, CP and NT

VIII. Estimate levels of oxidative stress makers (TPP, TAC, MDA and OSI) in ASD, CP and NT.

IX. Determine the relationship between levels of trace elements and oxidative stress markers in children with ASD, CP and NT

1.5. SIGNIFICANCE OF THE STUDY

Research into toxic and essential elements, neurotransmitters and markers of oxidative stress in neurodevelopmental disorders (ASD and CP) may improve the currently limited knowledge on the aetiology of ASD and provides information and rational approaches to the diagnosis, prevention and management of ASD and CP in this environment. Also, research into toxic and trace elements in occupationally exposed pregnant women may provide information on prenatal toxic and trace elements status involvement in the aetiopathogenesis of neurodevelopmental disorders (ASD and CP).

1.6 HYPOTHESES

1.6.1 Major Null Hypotheses

1.6.1.1 Cord blood toxic (Pb and Cd) and trace (Se, Zn, Cu, Mg and Ca) elements concentrations do not reflect maternal levels based on placental transfer mechanism.

1.6.1.2 Trace elements neither altered neurotransmitters and oxidative stress markers nor involved in the pathogenesis of neurodevelopmental disorders (ASD and CP) in children.

1.6.2 Alternate Hypotheses

1.6.2.1 Cord blood toxic (Pb and Cd) and trace (Se, Zn, Cu, Mg and Ca) elements concentrations reflected maternal levels based on placental transfer mechanism.

1.6.2.2 Toxic and essential elements altered neurotransmitters and oxidative markers and are involved in the aetiopathogenesis of neurodevelopmental disorders (ASD and CP) in children.

1.6.3 Sub-Hypotheses

1. There will be no significant differences in the environmental factors in occupationally exposed and unexposed pregnant women.
2. There will be no significant difference in socio-economic status between occupationally exposed and unexposed pregnant women.
3. There will be no significant differences in toxic metals (Pb, Cd) levels in exposed and unexposed pregnant women
4. There will be no significant differences in levels of essential elements (Cu, Zn, Ca,Mg, and Se) between occupationally exposed and unexposed pregnant women.
5. There will be no significant differences in cord blood essential elements (Se, Ca, Cu, Zn and Mg) concentrations between exposed and unexposed groups.
6. There will be no significant correlation in levels of toxic elements in maternal and cord blood
7. There will be no significant correlation in levels of essential elements in maternal and cord blood.
8. There will be no significant difference in socio-economic factors in ASD, CPand NT.
9. There will be no significant difference in medical and health issues among ASD,CP and NT
10. There will be no significant differences in environmental and household dustexposure factors among ASD, CP and NT.
11. There will be no difference that is significant in biodata variables of NDDs andNT.
12. There will be no significant differences in concentrations of toxic elements (Pb, Al,As, V) between children with NDDs and NT children.
13. There will be no significant differences in levels of essential elements (Mg, Ca, Zn, Se, Mn, Cu) levels between NDDs and NT children.
14. There will be no significant difference in neurotransmitters (glutamate, GABA,glutamine) levels between NDDs and NT children.
15. There will be no significant difference in levels of markers of oxidative stress` (TAC, MDA, OSI) between NDDs and NT children.
16. There will be no significant differences in biodata variables in ASD and NT.

17. There will be no significant differences in toxic metals (Pb, Al, As, V) concentrations in ASD and NT children.
18. There will be no significant difference in micronutrients (Mg, Ca, Zn, Se, Mn, Cu)levels between ASD and NT children.
19. There will be no significant differences in neurotransmitters (glutamate, GABA)levels in ASD and NT children.
20. There will be no significant difference in levels of oxidative stress markers (TAC, MDA,TPP) between children with ASD and NT.
21. There will be no significant differences in biodata variables between CP and NTchildren.
22. There will be no significant differences in toxic metals (Pb, Al, As, V) concentrations between children with CP and NT.
23. There will be no significant difference in micronutrients (Mg, Ca, Zn, Se, Mn, Cu)levels between children with CP and NT children.
24. There will be no significant differences in neurotransmitters (glutamate, GABA)levels in children with CP and NT children.
25. There will be no significant difference in levels of oxidative stress markers (TAC, MDA,OSI, TPP) between children with CP and NT children.
26. There will be no significant differences in biodata variables in ASD and CPchildren.
27. There will be no major variations in toxic elements (Pb, Al, and As) concentrations in children with ASD and CP.
28. There will be no significant difference in essential elements (Mg, Ca, V, Zn, Mn and Cu) concentrations between children with CP and the NT children.
29. There will be no significant differences in neurotransmitters (glutamine, glutamate and GABA) levels between children with ASD and those with CP.
30. There will be no significant difference in levels of oxidative stress markers (TAC, MDA,OSI and TPP) between children with ASD and those with CP.

CHAPTER TWO

LITERATURE REVIEW

2.1. Autism Spectrum Disorders (ASD)

2.1.1. Definition And Concept

Autism spectrum disorders (ASDs) are a range of neurological impairments evident by communication and social difficulties, and also some repetitive interests and behaviors (Siniscalco & Antonucci, 2013; Hodges *et al.*, 2020). The impairment can vary from moderate to severe in any of these dimensions; in extreme cases, such as in a low-functioning person who manifest as cognitively disabled, non-verbal, self-injurious and practically not able to communicate with even the closest members of the family. While a high-functioning autistic person may be expressive, have above-average cognitive ability, idiosyncratic areas of interest in which they tend to focus on social contact, and have formed close relationships with family, teachers, and some peers.

ASD is one of the disorders that are most devastating among the childhood disorders in terms of incidence, morbidity, end results, family consequences, and societal cost. ASD is recognized as one of the conditions of prenatal and postnatal brain growth disorders rather than an emotional disruption triggered by early attachment experiences, and mainly a genetic disorder involving several genes, necessitating a multidisciplinary approach to investigate the underlying mechanisms (Trevarthen & Delafield-Butt, 2013).

2.1.2. Historical Perspective

Paul E. Bleuler coined the word "autism" to describe the symptoms of mental illness called schizophrenia (Maatz *et al*., 2015; Park, *et al.*, 2015). Asperger borrowed Bleuler word "autistic" to characterize child psychology after the Greek word (autos) meaning self. He later observed and reported that four boys were not relating with peer group and never knew the meaning of the words- "respect" and "politeness," as well as respect for an adult's authority.

The boys often displayed stereotypical movement and behaviours that were out of the ordinary. Asperger coined the term "autistic psychopathy" to describe this pattern of actions, which is also known as Asperger's Syndrome (Asperger,1944; Park, *et al.*, 2015).

In his 1943 book, 'Autistic Disturbances of Affective Touch,' Leo Kanner was credited for identifying autistic children first. According to Kanner's publication in 1943, he reported behaviour and range of idiosyncrasies in both boys and girls. These, he said were different from anything illustrated before. This clinical description of the cases as portrayed by Kanner formed the basis of the three domains in which ASD is presently recognized; these include communication problems, social contact issues, restricted attitudes, and recurring interests (Atbaşoğlu, 2020). He was able to determine that the condition first manifested itself in childhood, distinguishing it from previous cases of schizophrenia and infant hysteria, while he also observed that the children tend to have average intelligence (Jablensky, 2010).

Other identified traits as described by Kanner are based on overarching features including marked social detachment, obsessive emphasis on sameness, language devoid of inter-personal communicative skills. Kanner later called this constellation of signs and symptoms "early infantile autism (Park *et al.,* 2015). His use of terms was intended to highlight the onset date as well as the important feature of restricted accessibility (Kanner, 1965). However, Kanner's clinical description of ASD then remained the hallmark of the disease by many researchers till date.

2.1.3. Diagnosis

Although in terms of symptom classification and evaluation development, autistic disorder received the most publicity (Matson *et al*., 2007), uniformity even in the clinical picture of autism syndrome remains an issue across various medical communities. Thus, different categories of diagnostic variations were created and available. Some were fashioned after the original description by Kanner while others were amalgamation of other emerging pictures of the disorder with the original picture as painted by Kanner and Asperger. These include Diagnostic and Statistical Manual of Mental Disorders (DSM5) as well as its various modifications as proposed by APA (Regier *et al*., 2013). However, the emergence of International Classification of Diseases (ICD) as a certified diagnostic manual to which APA and DSM have largely been aligned remains the most

popular of all the categorization (APA, 2013).

Current Criteria

The diagnostic clinical criteria for ASD have been collated in 5 different categories/stages (DSM 1-5); the 5 new diagnostic criteria (DSM5) are as discussed below:

To satisfy the ASD diagnosis according to DSM-5, the following criteria must be fulfilled by individual at the time of diagnosis or by history:

1. Social communication deficit must be evident and persistent
2. Impairment in interaction across contexts
    (a) Social interaction impairment
    (b) Impairment of social contact nonverbal communicative actions
    (c) Difficulty in forming and sustaining friendship
3. Two, at least out of the following restricted interest and repetitive habits of actions or behaviors should be shown by the individual:
    (a) Repeated or stereotyped voice, motor movements, or object usage
    (b) Too much adherence to habits, ritualized verbal or nonverbal behaviour patterns, or a strong fear of change are all examples of a strong resistance to change
    (c) Abnormally limited, fixated, severe, or concentrated habits
    (d) Hyper- or hypo-reactivity to sensory stimuli, as well as an unusual fascination with sensory elements of the environment.
4. All of the symptoms are visible in early life, but not fully developed until social expectations exceed limited abilities.
5. The individual's daily functioning must be limited and impaired as a result of the symptoms.

Due to the lack of biomarkers, autism is still diagnosed using developmental background, behavioural findings, and administrating standardized tests (Reichow *et al.*, 2008). While DSM-IV and DSM-V diagnostic criteria are based on behavioural features (Fig. 2.1), they differ in their categorization of symptoms (Table 2.1 & Fig. 2.2)

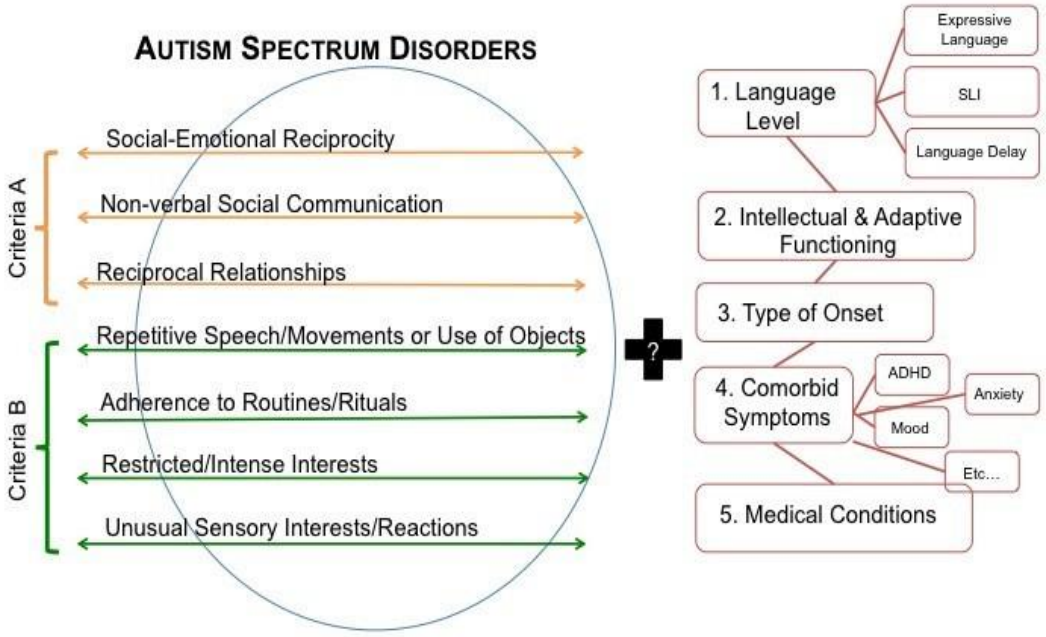


Figure 2.1: Criteria and characteristic features of ASD (Grzadzinski, R., Huerta, M., & Lord, C., 2013).

Table 2.1: Neurodevelopmental Disorders Assessments Using DSM-4 and DSM-5 (Froehlich & Fung, 2012)

DSM-IV	DSM-5
The disorder includes: Asperger disorder, Autism disorder, Childhood disintegrative disorder, persuasive developmental disorder not otherwise specified	Autism spectrum disorder
Mental retardation	Intellectual disability
Rett disorder	Rett not included
No social communication equivalent in DSM-IV diagnosis	Social communication disorder
Expressive language disorder	Late language emergence or language impairment
Phonological dysfunction	Speech sound dysfunction
Reading impairment	Reading, writing and spelling impairment (Dyslexia)
Mathematics disorder	Mathematics disorder (Dyscalculia)
Mixed repetitive-expressive language impairment	No equivalent in DSM-5 diagnosis
Stuttering	Childhood onset fluency impairment

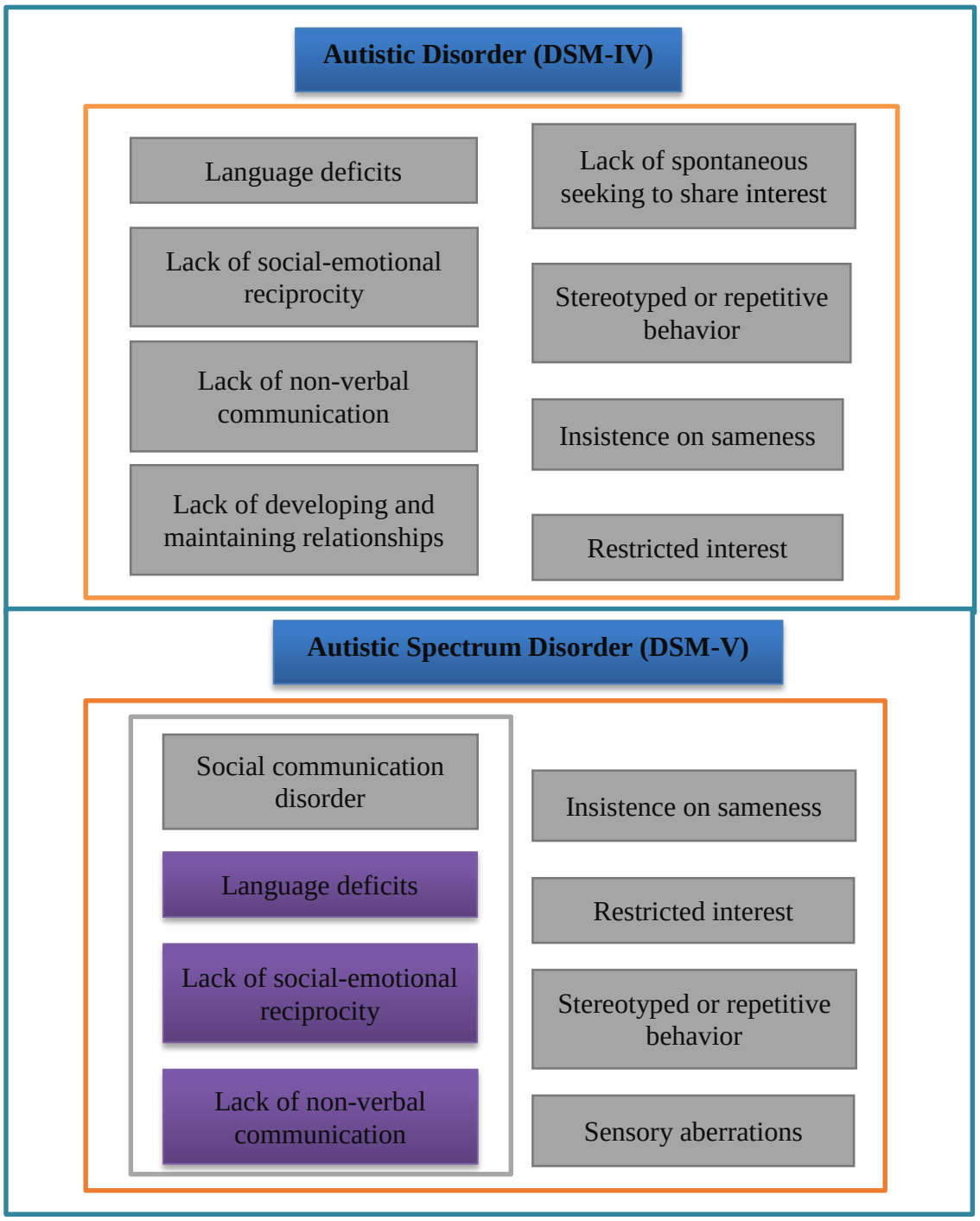


Figure 2.2: DSM-4 and DSM-5 symptom domains used to define ASD (Froehlich & Fung, 2012)

2.1.4. Epidemiology

The incidence of autism worldwide and even in the developed world is not very clear; the reasons may be largely due to lack of surveillance and non-uniform in its distribution. It is therefore not well understood if the disorders incidence is rising or not. However, most reports of incidence of autism are from the US. Autism was thought to affect 2-20 persons out of 10,000 in the 1960s and 1970s. Almost all epidemiologic research conducted prior to 1985 reflected results in this range (Gillberg & Wing 1999). However, since 1985, 10 of 11 researches done outside of USA have found incidence of 9 per 10,000 or higher. The most detailed of other studies with respect to case findings reported rates of > 20 per 10,000 children (Gillberg & Wing, 1999). These studies were performed on small populations.

A longitudinal study on the incidence of autism reported a prevalence of 4 in 10,000 in 1980; 7.6 in 10,000 in 1984 and 11.5 in 10,000 in 1988 (Gillberg & Wing, 1999). Autism was then projected to be growing at a rate of 3.8 percent per year, based on global epidemiologic studies. Although it is unclear if the apparent rise in prevalence is due to using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) for improved monitoring or a real increase. Infact, the state of California announced a 210 percent increase in the autism population receiving services through its regional centers in April 1999 (CDDS, 1999). The prevalence of 3.8% per annum reported earlier may be subjective, considering the 60% increase in ASD cases for the state of California alone among children attending a regional centre for autism between 1987 and 1998. This has led the State to priorities autism research (Baxter *et al*., *2015*).

The occurring rates of other countries' ASDs vary and were reported to be 0.02% in Norway, 0.9 % in South Korea (Sponheim & Skjeldal, 1998), 0.06 percent in Venezuela, and 1.7% in South Korea (Kim *et al*., 2011). The research methods employed in some of these studies are highly complex, making it difficult to compare results so as to obtain distribution data for ASD (Baxter *et al.,* 2015). The observed increase in prevalence was not limited to the developed world alone. In Nigeria, the prevalence was said to be 0.8% in prevalence study in 2011(Bakare *et al.,* 2011), 2.3% in hospital-based study in 2014 (Lagunju *et al.,* 2014) and 2.9% in 2016 (Chinawa *et al.,* 2016).

2.1.5. Prevalence

ASD, known as an uncommon condition, has come to the forefront of public consciousness because of its increasing incidence. Since the 1960s, reported prevalence rates have been gradually increasing, although it is unclear to what degree this reflects areal rise in incidence or improved understanding of ASD and diagnosis. Researchers who access academic and medical data discovered significantly increased incidence compared to those using medical records only. Some researchers concluded that the ASD high incidence is due to improvements in autism diagnosis practices. For example, the chances of a child being diagnosed with ASD increase when they exhibit an early intellectual disability (ID) (Neggers, 2014). Fombonne reported in 2002 that the prevalence of ASD from an in-depth English language studies in 1966 and 2001 was 10 in 10,000 and 27.5 in 10,000, respectively. He speculated, however, that if research published at the time (in the early 2000s) was repeated, the incidence may be closer to 60-70 in 10,000. In the year 2007, CDC in US registered higher prevalence of 1 in 152 children (White *et al*., 2009).

ASD affects one out of every 150 children, according to Matson & Shoemaker (2009). Since the first epidemiologic trials in the late 60s and early 70s, the global incidence of autism has risen twenty-fold to thirty-fold. At the time, European studies estimated that 1in 2,500 children in the population had the disease (Lyall *et al*., 2017), and the incidence estimates from a comprehensive survey in the 2000s ranged from 1% to 2% of all children (Schaefer & Mendelsohn, 2013). Extrinsic factors such as increased understanding and identification, as well as improvements in diagnostic practice or service availability, have been linked to the apparent prevalence changes (Rossignol and Frye, 2012). In 2010, in the USA, ASD prevalence was estimated to be 14.7 per 1,000 (1 in 68) (1.47%) in 8-year-old children (CDC. 2014; Baio *et al.,* 2018; Chiarotti & Venerosi, 2020) but the current estimate (Fig. 2.3) proposed a 15 percent rise in prevalence rates, making it 1 in 59 (1.69%) (CDC, 2018; Guifeng *et al.,* 2018).

ASD incidence differs based on gender and race according to research. According to the CDC, males had a prevalence of 18.4 per 1,000 (1 in 54) (1.84 percent) and females had a prevalence of 4.0 per 1,000 (1 in 252) (0.4 percent). The prevalence rate of 12 per 1,000 (1.4 percent) found in non-Hispanic white children was significantly higher than 10.2 per 1,000 (0.8 percent) in non-Hispanic black children and 7.9 per 1,000 (0.9 percent) in Hispanic children (CDC, 2013). Given the lack of clearly established

differences in ASD risk factors among these groups, inequalities in prevalence rates predict that Hispanic and non-Hispanic black children are under-identified (ACE, 2015; Maenner *et al*., 2020).

In Nigeria, there is limited information on the prevalence of ASD; however, the prevalence is on the increase too, comparing 0.8% by Bakare and Munir (2011) to 2.3% by Lagunju *et al.,* (2014) in their hospital-based studies. In summary, there are enough reasons and evidences to ascertain that the prevalence of ASD is actually on the increase globally; what perhaps is in contention is the mode of diagnosis to have fairly reliable and reproducible prevalence rate globally.

2.1.6. Aetiology

As far as the current knowledge about the aetiology of autism is concerned, the physiological disorders associated with autism are induced by a combination of hereditary susceptibility and environmental insult. The evidence proves that ASD is caused by irregular regulation in several epigenetic modification mechanisms, regional brain structural changes and dysfunctional neural networks. ASD is largely a heritable disorder, according to human population studies (Lyall *et al.,* 2017). Other factors (especially environmental) are very likely to be involved in the manifestations of the disorder. The probability of ASD occurring in another child from same family that has a child with ASD is 50 -150 times higher on the average in comparison to the probability general population (Tordjman *et al.,* 2014). The heritability in ASD is estimated to be 90% in monozygotic and dizygotic twins (Tick et al., 2016). According to studies, ASD show phenotypic plasticity, with three to fifteen genes per person and diverse gene–environment interactions (Sambandan *et al*., 2008).

The location of susceptibility genes has been mapped using genome-wide linkage scans. At least 12 genome scans have been published, albeit variations in genetic analysis methods, genetic techniques and research techniques. Although few results met the statistical significance criterion set forth by Lander & Kruglyak (1995), on chromosomes 2q, 7q, and 16p, there is a fusion of possible interconnections (IMGSAC, 2001). About 100 gene variants were tested as potential choices (Wassink *et al*., 2004), but only a few has been confirmed. ENGRAILED 2, a cerebellar developmental patterning gene, has been linked to ASD in three separate groups (Genestine *et al*., 2015). The UBE3A gene (which causes Angelman's syndrome) on chromosome 15q11–

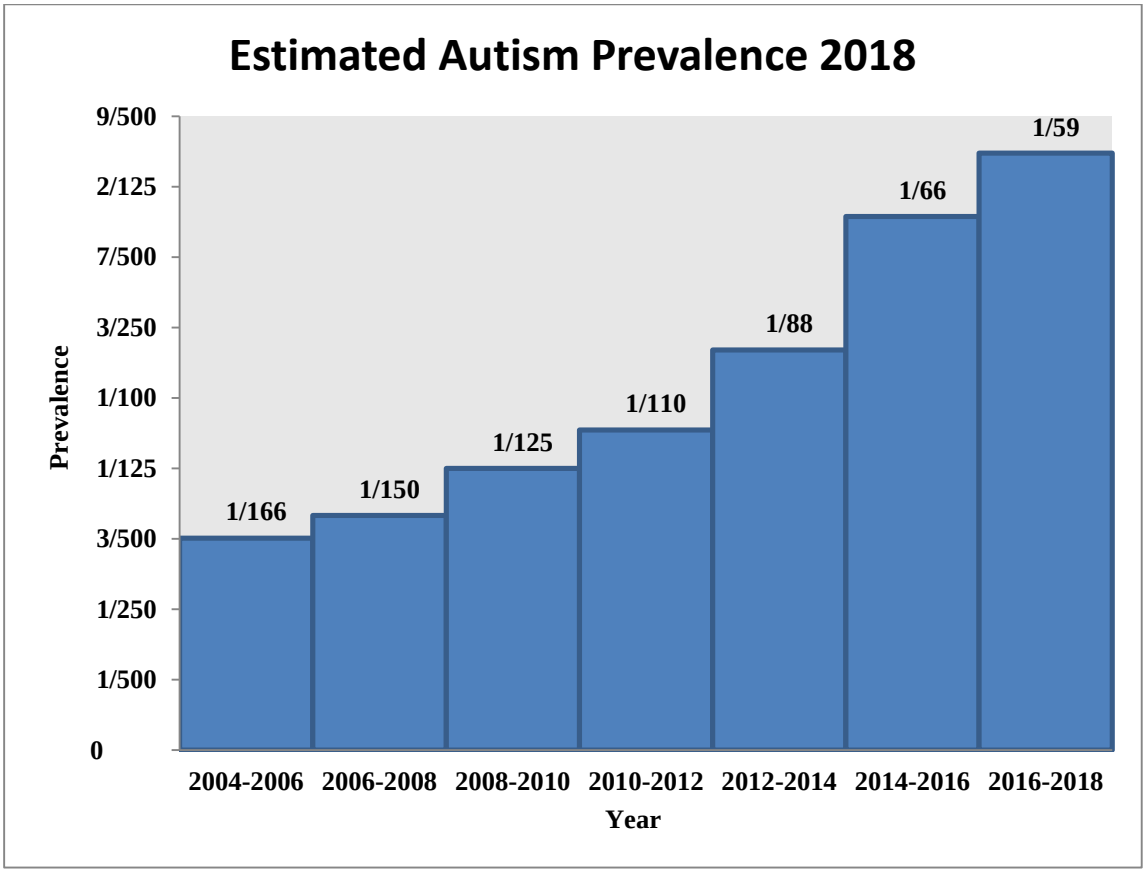


Figure 2.3: Estimated Autism Prevalence (CDC, 2018).

13, multiple GABA system genes, as well as the serotonin transporter gene on chromosome 17q are all promising candidate genes (Devlin *et al*., 2005). The identification of genomic regions that could include susceptibility genes has also been supported by the analysis of ASD cases with rare chromosomal defects (Vorstman *et al.*, 2006). Relevant Autism genetic traits (endo-phenotypes, such as cognitive deficits may be more genetically helpful than an ASD diagnosis (Geschwind, 2011). Aside from the NIH's earlier study on the role of the environment in ASD, there has also been a lot of focus on the role of other environmental factors (London, 2000). So many prenatal exposures are being attributed to ASD, include thalidomide, some viral diseases, and prenatal anticonvulsants, particularly valproic acid (Christensen *et al*., 2013). Even though the percentage cases accounted for is small, these factors can cause genetic susceptibility to be disrupted, increasing the risk of ASD. According to some evidence, a history relating to disorders of autoimmune and the existence of neural antigens autoantibodies in family are both immune factors that may play a significant role (Van de Water, 2004). Recently due to industrialization, many other environmental factors have been implicated as possible agents that may interact with the genes especially in susceptible subjects, leading ultimately to the pathogenesis of ASD. Hence, in the study of ASD pathogenesis, the emphasis is gradually shifting towards epigenetic modifications (Fig 2.4).

2.1.7. Causes

While there are various hypotheses, there is no single accepted cause of ASDs at this time. It is becoming evident that ASD is triggered by a dynamic interaction of multiple variables (genes, environment, and central nervous system), and that it is not an etiologically homogeneous condition. In other words, there are possibly several sub-types of ASDs, each with its own etiology. While genetics has also been implicated, it does not completely explain the situation or the recent increase in cases reported. According to research, diagnosis of one identical twin with ASD proves the other twin has a 30-40% risk of developing the disorder. Non-identical twins rarely have this degree of concordance (Frazier *et al*., 2014). When a broader description of ASD is utilized, there is likelihood that the chance for identical twins increases to 90% and 10% for non-identical twins (Chaste & Leboyer, 2012). When another sibling has already been diagnosed with ASD, the chances of getting an ASD diagnosis are projected to be between 2 and 14 percent, which is a 10- to 20-fold rise in prevalence compared to the

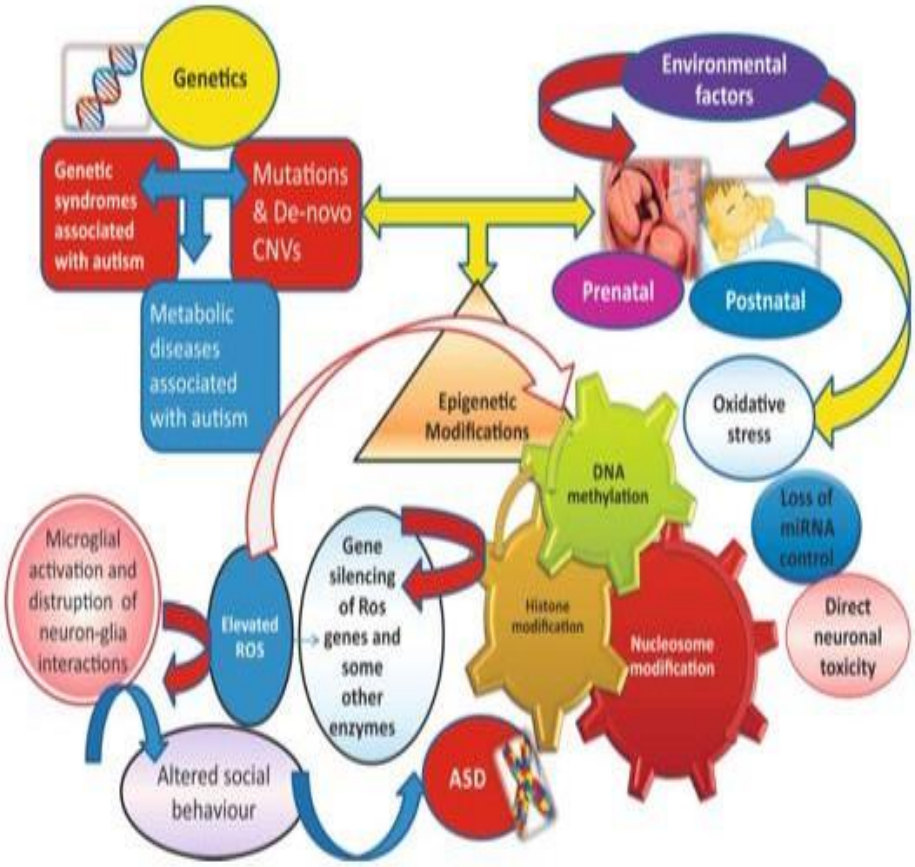


Figure 2.4: Etiology of Autism Spectrum Disorders through epigenetics (Bhandari, R., Paliwal, J. K., & Kuhad, A., 2020).

general population (Neggers, 2014).

There is not a single gene identified as the cause of ASD but most genetic studies suggest that multiple genes are involved (Yoo, 2015). According to genetic research, at least 40% of ASD cases may be linked to the environmental factor (Chaste & Leboyer, 2012). Evidence also abounds that a subset of children with ASD (20-30%) demonstrate ability regression between 18 and 24 months after seemingly initial normal growth, but consistent developmental milestones delay were reported in other ASD children (Al Backer, 2015). According to a few studies, certain cases of ASD have been related to maternal prenatal exposure to some pathogens and contaminants. They accounted for a relatively small percentage of the disorders (Karimi *et al*., 2017). There has been proof that the protectant thimerosol, which is used in vaccines, is connected to a certain case, though the proof for the majority of these is ambiguous (IOMISRC, 2001).

Environmental factors (such as heavy metals, polychlorinated chemicals, and PDBEs) are becoming common hypotheses among researchers. The role of immune function in the aetiology of ASD is also gaining popularity with the possibility that the contaminants such as car exhaust, organophosphates, and organochlorine (all of which are commonly found in our environment) are to be blamed for the reported brain inflammation in NDDs (Landrigan *et al.,* 2012). A typical brain growth is commonly believed to be at the root of the ASD signs that can be seen; however, the interaction between environmental toxicants and the genes to impair brain function is still less defined (Fig. 2.5). Although social and behavioural signs of ASD will not manifest till around eighteen months, it is clear that such effects on brain development can be linked directly to pre- or post-natal period. The origin of the variations in the brain is still unknown. Some brain regions like cerebellum, subcortical amygdala, frontal lobes, temporal lobes and hippocampus have demonstrated distinct development in studies (Pandya *et al*., 2012). Owing to scarcity of data, analytical difficulties and contradictory results, detailed conclusions about the particular affected brain regions or developmental mechanisms that lead to the observed brain difference have yet to be drawn. While some look at interaction patterns within the brain areas rather than individual loci (Courchesne & Pierce, 2005), others focused on particular neuron (mirror neurons) or neuronal activation patterns.

There is currently no treatment for Autism due to a lack of scientific evidence on its

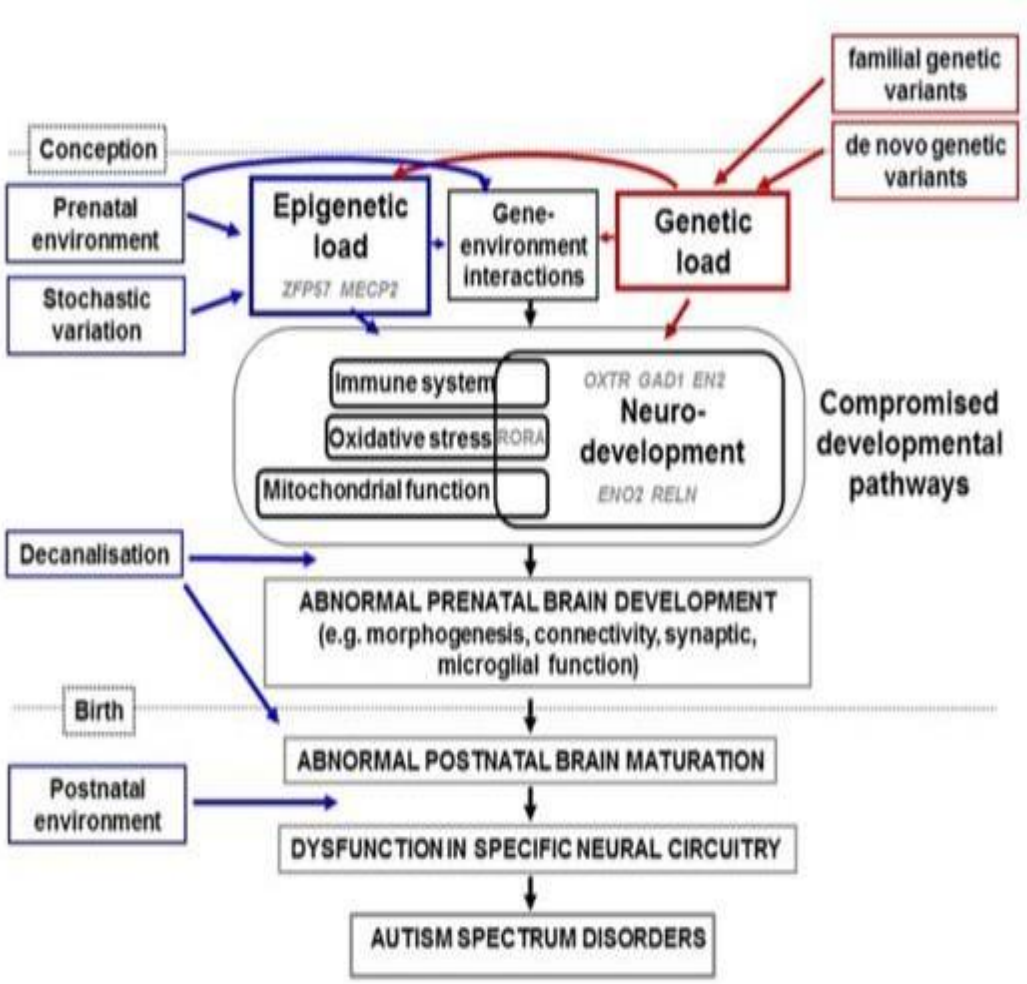


Figure 2.5: Environment and genetic interaction combine in the aetiology of Autism Spectrum Disorders (Loke, Y. J., Hannan, A. J., & Craig, J. M., 2015)

causes. Several studies, however, point to a close correlation between environmental factors and the development of ASD. This epigenetic perspective on the pathophysiology of this condition is gaining traction (Landrigan, *et al*., 2012).

2.2. Cerebral Palsy (CP)

Cerebral palsy is also a neurodevelopment problem, first identified in the 1840s by William Little. With degrees of impairment varying between moderate disability to serious impairment, the disorder presents diagnostic and clinical difficulties to physicians. It is often linked to a number of co-morbid disorders (Gedam *et al*., 2014; Novak, 2014). Cerebral palsy is a disorder of the nervous system that causes motor weakness as well as physical and mental dysfunction. In 2001, about 764,000 US children and adults were diagnosed of cerebral palsy; based on the figure from United States Cerebral Palsy Foundation. (UCP, 2005). Every year in the United States, almost 8,000 new born as well as over 1,200 infants were reported as cases of cerebral palsy (Krigger, 2006). It is important to differentiate CP from ASD using biomarkers.

2.2.1. Definition

Cerebral palsy (CP), a known diverse chronic nervous disease, results from non-progressive brain injury (Rana *et al*., 2017). The word "palsy" indicates a lack of muscle coordination, whereas "cerebral" means "brain." CP is primarily a posture and motion disorder. CP is defined as an overarching concept encompassing plenty of non-progressive, but evolving, motor dysfunction syndromes triggered by lesions or defects in early stage of brain development (shona *et al*., 2018). The injury or malformation in CP occurs in the developing nervous system, affecting motor control and body posture before, during, and soon after birth (Schimdt *et al*., 2017). It can manifest as a persistent encephalopathy, the clinical appearance varies over time due to the central nervous system's developmental plasticity and maturation, despite the fact that the initial lesion, anomaly, or injury is static. (Chitra & Nandini, 2005).

2.2.2. Prevalence

The global incidence of about 2 to 2.5 in 1000 live babies was reported in CP according to Rosen and Dickinson (1992). When Little first identified cerebral palsy, he blamed it on birth trauma, and this belief has continued for decades. The occurrence of CP is not reduced in spite of recent improvement in neonatal management and obstetric care (Nelson, 2003). There is limited information globally on the incidence of CP; although,

data obtained largely from the US showed it varies from 3.3 per 1000 in Wisconsin to 3.7 per 1000 in Alabama and 3.8 per 1000in Georgia. In comparison to girls and Hispanic, boys and black non-Hispanic have the highest CP cases. It has also been discovered that children of low and middle-income households had a greater incidence than children of high-income households (Winter *et al.*, 2002). On the contrary, despite a decrease in infant mortality, the number of individuals and seriousness of cerebral palsy has increased. Premature babies have higher incidence rates compared to full-term babies (Vincer *et al.*, 2014; Rana *et al*., 2017). Although complications during child birth has been proposed as possible basis of CP; however, birth asphyxia or obstetric problems cannot be attributed solely for CP development in the majority of term infants (MacLennan, 1999).

2.2.3. Aetiology

Cerebral palsy is a non-reversible brain problem resulting from trauma to the brain even before the completion of brain development. Since brain development takes place throughout the first 2 years of life, brain damage that occurs during *in-utero*, perinatal, or after birth may cause cerebral palsy (UCP, 2005). CP has a multifactorial aetiology. Congenital, hereditary, inflammatory, bacterial, anoxic, traumatic, and metabolic factors are among the possibilities (Kurt, 2016). Prenatal, perinatal, or postnatal trauma to the not-fully developed brain can occur; *in-utero* injury accounts for 75 percent to 80 percent of cases, with fewer than 10% attributable to major birth trauma or other factors (Fig. 2.6) (Hacker *et al.*, 2016).

Premature births and accompanied Low birth weight was reported as the most prevalent risk factors of CP because the risk of CP rises as birth weight and pregnancy age decrease (CDC, 2013). Cerebral palsy affects 10–18% of babies weighing less than 1000 grams at birth (Reddihough & Collins 2003). Children born prematurely or at term are more likely to develop CP (Michael, 2004). Despite the fact that term babies have little risk (Michael, 2004), termed births are responsible for the majority of all the births with CP. It was reported that among the children born with CP, up to 12 percent was termed babies and 28 percent was premature babies (Wu & Colford, 2000; Wu *et al*., 2006). Another risk factor of developing CP is Cystic Periventricular Leukomalacia (CPVL) which accounts for 60% to 100% of cases (Wu *et al*., 2006). The possible mechanism of CP development from CPVL is shown in Fig. 2.7. Placental complications, mutagenic contaminants, intrauterine infections, multiple births and

maternal illness like mental disorders, seizures, or thyroid issues are among the prenatal risk factors of CP. Prenatally developed cerebral palsy accounts for 70 to 80 percent of all cases, with the causes still unknown. Twins and triplets have a higher rate of CP than singletons. Perinatal risk factors include infections, intracranial hemorrhage, seizures, low blood glucose, elevated blood bilirubin, and severe fetal distress (Drougia *et al.*, 2007). Brain damage may occur before, during, or after birth. According to a research, prenatal insult is responsible for 75-80% of confirmed cases, whereas severe birth trauma or asphyxia is responsible for only 10% (Jain *et al*., 2015).

Another possible cause of hemiplegic CP in many babies has been described as perinatal arterial ischemic stroke. Around 6% of patients with congenital cerebral palsy are thought to be affected by birth complications, such as asphyxia (Taylor, 2001).

Toxaemia, auto-immune, encephalopathy, meningitis, trauma events like drowning are all postnatal causes (Chitra & Nandini, 2005). . The is a link between coagulopathies that cause cerebral infarction and CP, particularly hemiplegic CP. About 12–21 percent of all CP cases are caused by postnatal factors. However, the origin of CP is unknown in a significant number of cases (Chitra & Nandini, 2005).

2.2.4. Associated Deficits In CP and Complications

Cerebral Palsy has been related to a range of neurological conditions and complications, some of these make the case an ideal positive model in understanding the other rare neurodevelopmental disease, ASD. These associated disorders/complications are:

- Mental retardation (MR) affects up to 60% of people with CP (Türkoğlu *et al.*, 2017). Spastic quadriplegia causes more cognitive dysfunction in children (Patel *et al.*, 2020).
- About 28% of the children with CP have visual impairments and defect of ocular motility visual disabilities, such as refractive errors, are becoming more common. Visual perceptual issues are more prevalent in CP caused by periventricular leukomalacia.
- Hearing loss affects about 12% of cerebral palsy children. This is associated to extremely low birth weight, jaundice that causes yellowness of the brain or severe hypoxic ischemic attacks (Dani *et al.*, 2021).

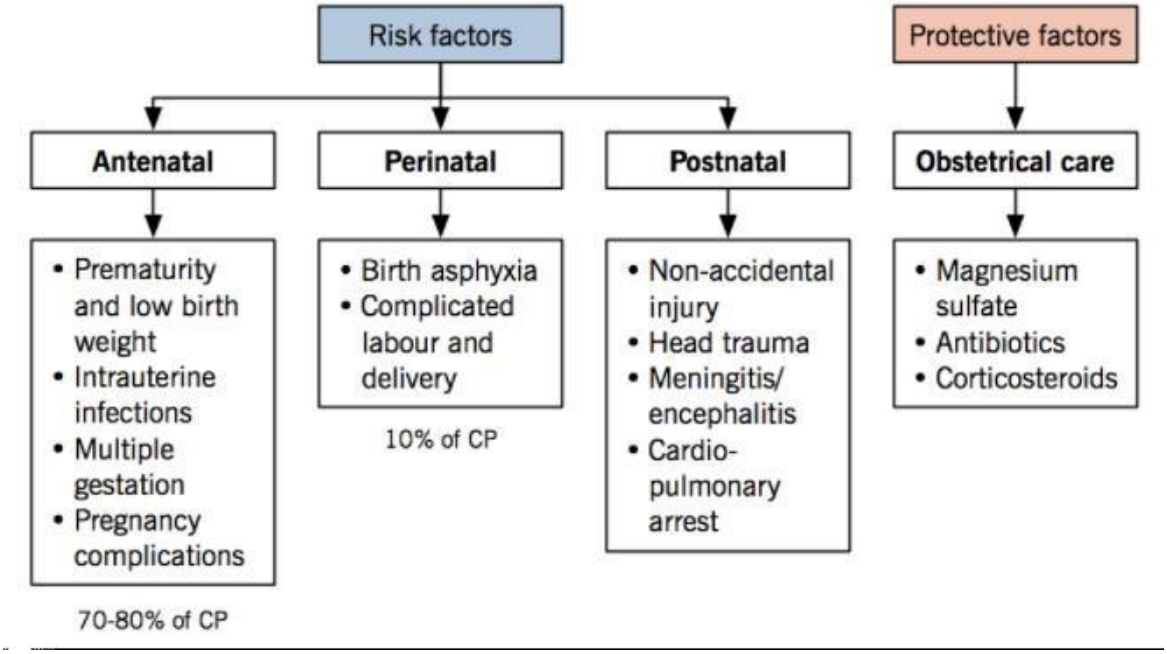


Figure 2.6: Cerebral palsy risk factors (Hacker, N. F., Gambone, J. C., & Hobel, C. J., 2015)

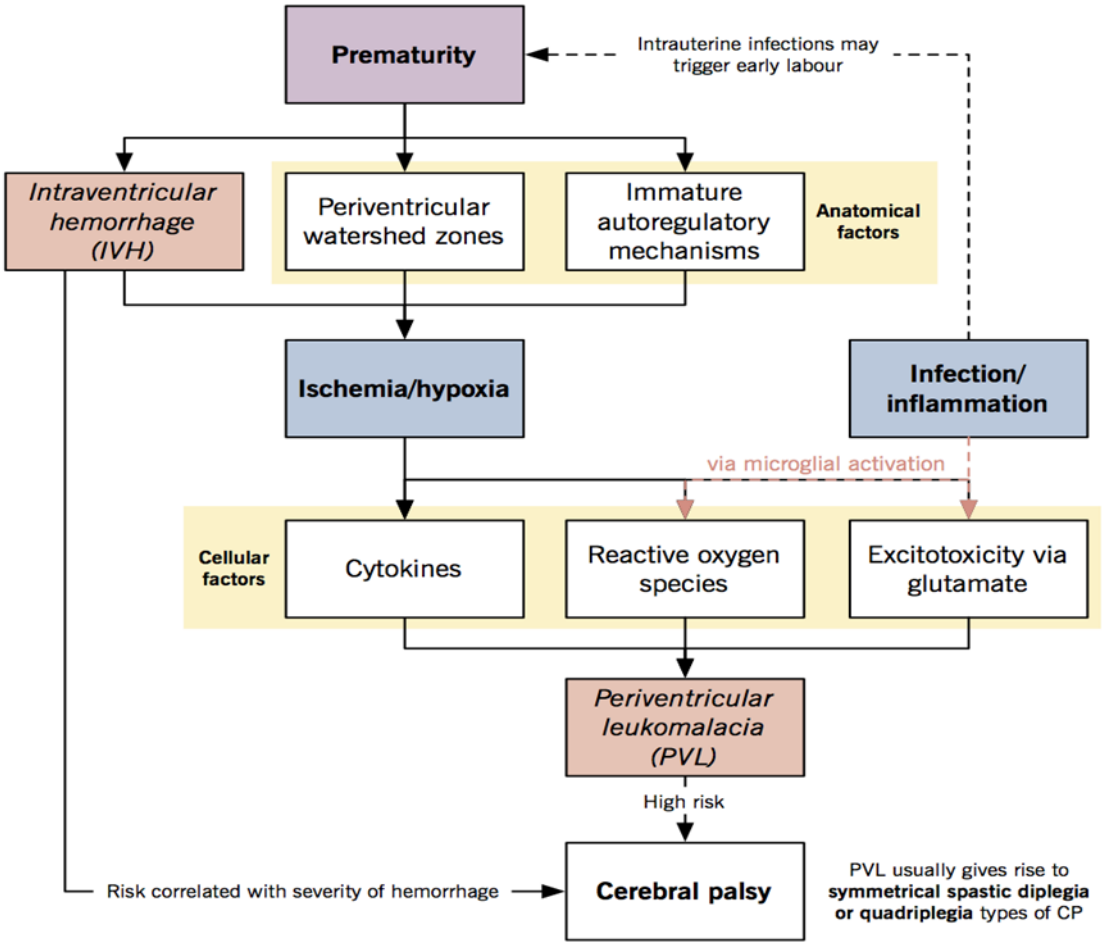


Figure 2.7: Development of Cerebral Palsy from Periventricular Leukomalacia (Saunders, R. A., 2009).

- Epilepsy is a very common co-morbidity in cerebral palsy. It occurs in 35 to 62 percent of children diagnosed of CP. Epilepsy is more common in persons that have spastic quadriplegia (50 - 94 percent) or hemiplegia (30 percent) than in those with diplegia CP (16 to 27 percent). Singhi and his colleagues in a survey done in India reported that 35 percent of people were found to have epilepsy (Singhi *et al.,* 2003). Seizures were present in 66% of spastic hemiplegia, while 43% had spastic quadriplegia and spastic diplegia accounted for 16% (Mesraoua *et al*., 2019).
- Speech and Language Disorders: Bilateral corticobulbar and/or motor dysfunctions impair speech in people with CP. Language deficits, both receptive and verbal, are typical in people with mental retardation. Articulation problems and stuttering affect 38 percent of children with cerebral palsy. Motor issues such as difficulty eating, swallowing problems, and drooling are also prevalent.This causes dietary issues that affect physical development (van den Engel-Hoek et al., 2015). Behavioral issues are also well-documented. In children with CP, abnormalities in positioning and technical sensations are common. Anxiety, depression behavioural disorders, attention as well as hyperkinesis were reported in 61 percent of the 6 percent of 10-year-old children that had hemiplegic CP (Srivastava *et al.,* 1992; Ojturk *et al.,* 2002). For the CP child, the related deficit can be more devastating than the motor problem.

2.3. The Roles of Essential and Toxic Elements

Metals are electropositive elements with low ionization energies that have a bright luster, are heavy, and have the ability to resonate sound. Metals except mercury are solid under normal conditions and are also heat and electricity conductors. Metals are just like organic molecules which serves as important ingredients in life. The divalent magnesium and calcium ions, for example, play critical regulatory roles in cells (Fig. 2.8) (Jaishankar *et al.,* 2014).

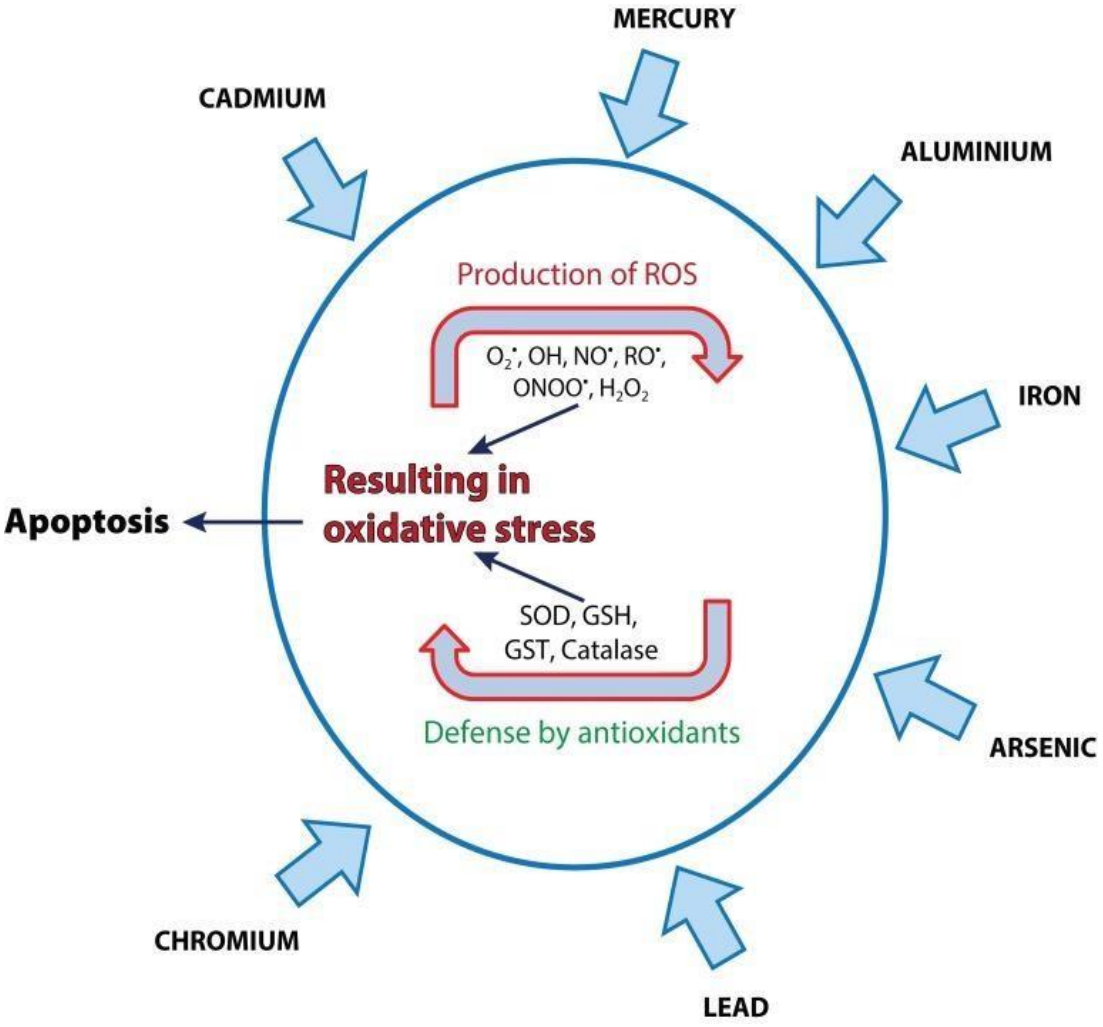


Figure 2.8: The impact of heavy metals on a cell, as well as the delicate balance between ROS generation and antioxidant defense (Jaishankar *et al*., 2014).

Essential Trace Elements

A number of metals were reported to be involved in health and diseases; they could broadly be classified as macro and micro elements. The **micro elements** are those that are required in micro and probably nano amounts. The micro elements can further be divided into toxic and trace elements. On the other hand, the **macro elements** are those that the body requires in milligram amounts. These include calcium, magnesium, sodium and potassium. They are all required in milligram quantities and are very essential for many metabolic functions of the body. The deficiency of some of these trace elements, particularly during maternal infection, has been associated with neurodevelopmental consequences in children (Fig. 2.9) (Labouesse *et al.,* 2015).

2.3.1. Calcium (Ca)

The chemical cation element "calcium (Ca)" is found in the human body in the highest concentration. It belongs to the group II elements on the periodic table (see Fig. 2.10). It is thus an active cation with a valence of 2 (Leadbeater, 2019). It is the most available cation in the body, but it is often found as a salt or phosphorus complex in the body (Shaker & Deftos, 2018). Calcium phosphate is formed when calcium interacts with phosphorus to create the hard, dense product that makes up bone and teeth. Calcium is essential for many metabolic functions of the body. It is the main skeletal frame of the body; hence, over 90% of body calcium resides in the bone and constitute the body calcium repository that essentially maintain blood calcium level (Ross *et al*., 2011). It is involved in blood coagulation and normal neuromuscular function. Because of the diverse functions of Ca in the body, it is available in two major types: total calcium as well as ionized calcium (Ross *et al.,* 2011).

The total calcium fraction is associated with its skeletal function, while ionized form is associated with various metabolic activities like enzymes activation, nerve impulses and muscle contractions (Ross *et al.,* 2011). Although the ionized fraction is a lot smaller in comparison to other fraction, the pathology of its deficiency manifests more in human health; for example, the importance of calcium in the initiation of neuromuscular as well as metabolic functions have been well documented (Committee to Review Dietary Reference Intakes, 2010). Also, calcium has also been implicated in the circulatory system, maintenance of osmolality of the extracellular fluid, muscle tonicity, intracellular signaling and hormonal secretion (Risteli *et al.,* 2012; Weaver, 2012).

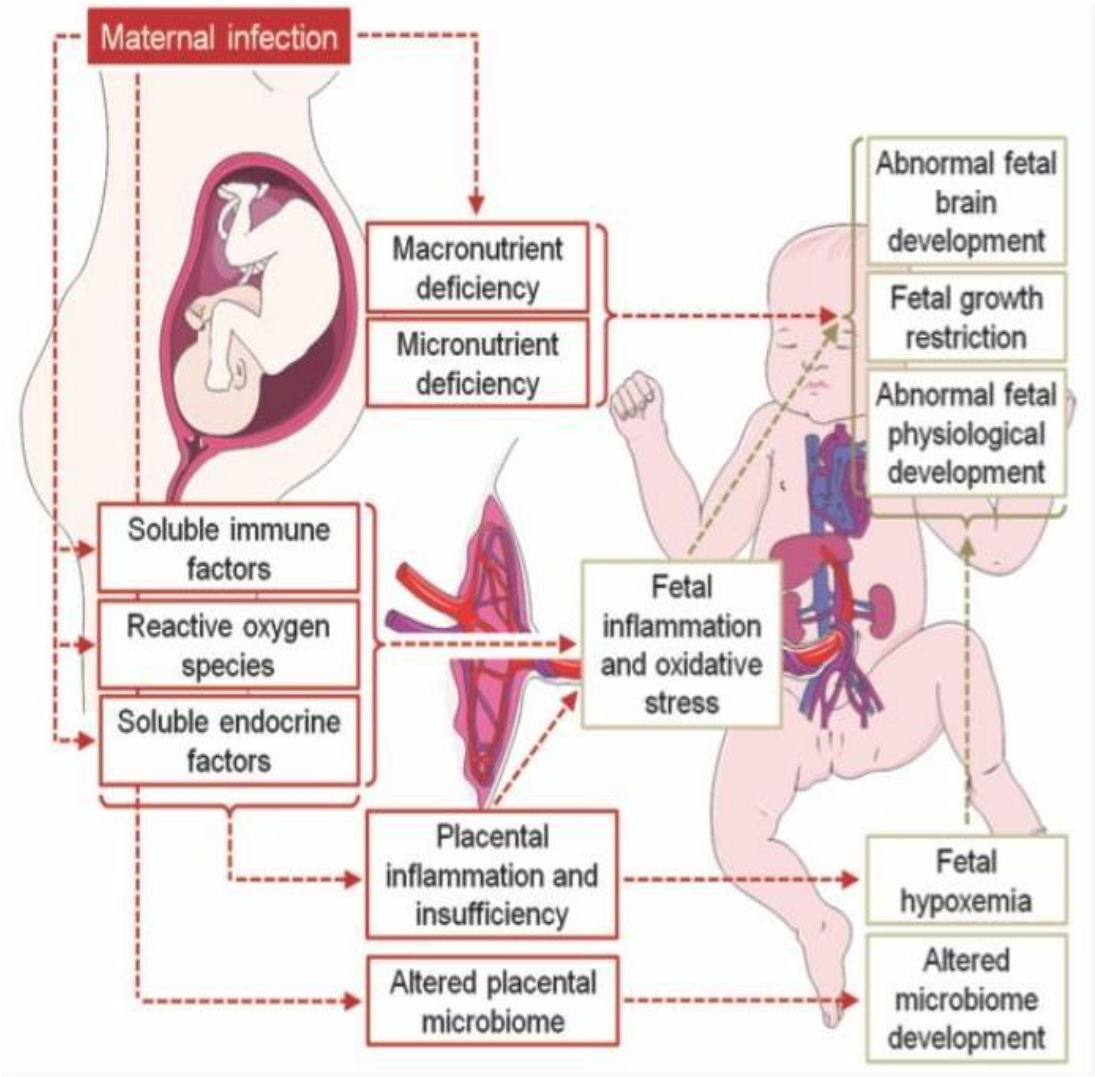


Figure 2.9: Maternal infection and trace elements in the aetiology of Neuro-Developmental Disorders (Labouesse, M. A., Langhans, W., & Meyer, U. (2015).

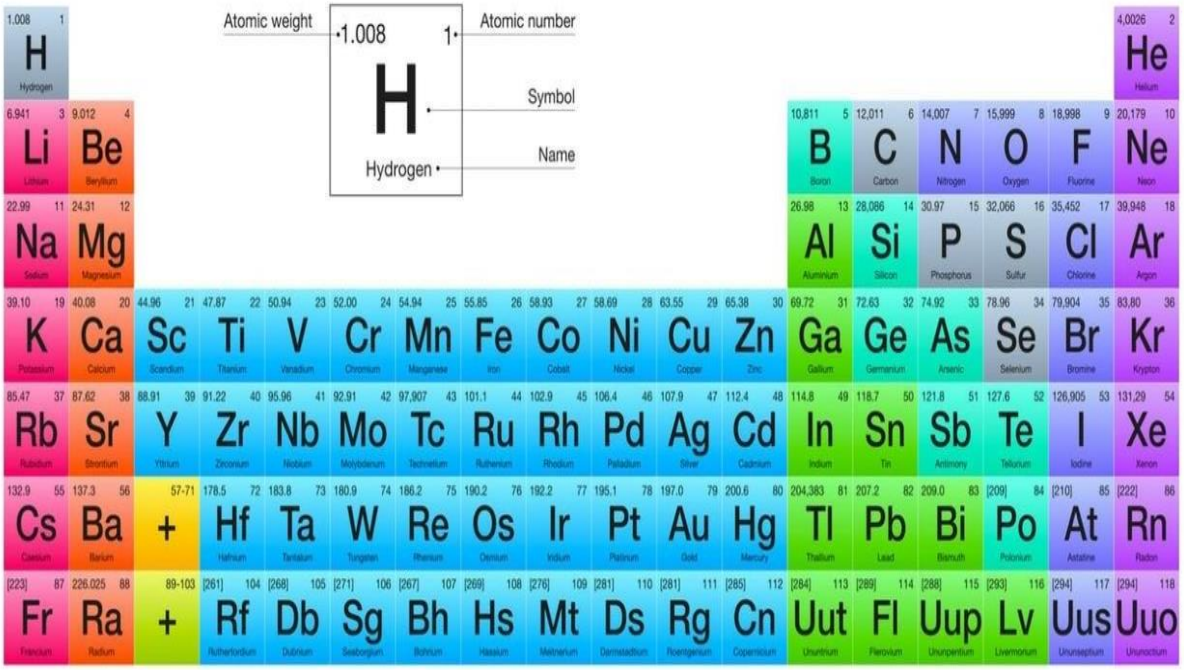


Figure 2.10: Periodic Table of Elements (Leadbeater, N. 2019)

2.3.1.1. Sources

Food is the main source of calcium. Most foods contain small quantities of calcium, but only a few contain significant amounts. Milk and milk products, especially cheese, are the best sources of ingested calcium. It is also gotten from bones and dietary supplements (NIH, 2019). It can also be obtained from fruits, vegetables, fish and other foods. The importance of calcium in metabolic processes has drawn attention to the various sources of the metal. This is imperative because most of the dietary sources of calcium as stated above contained minimum amount of calcium. Hence, calcium supplement is widely used by the elderly, particularly post-menopausal women whose common pathology is osteoporosis (Li *et al*., 2018; NIH, 2019).

2.3.1.2. Calcium Metabolism

Body calcium is primarily obtained from dietary sources, i.e., digested food that is absorbed into the bloodstream through the gut wall (Gurr, 1999). Calcium absorption takes place at the intestinal mucosa in two ways: transcellularly via an active transport and extracellularly via passive diffusion. Calcitriol and the vitamin D-receptor in the intestine are needed for active transport (VDR) (Ross et al., 2011). Transcellular pathway is activated by calcitriol, which largely accounts for calcium absorption at low to moderate intake levels (Ross *et al.,* 2011). Calcium transfer between the mucosal cells is known as passive diffusion or paracellular uptake, and luminal serosalelectrochemical gradients are needed for this. Passive diffusion is more common with high calcium intakes (high luminal concentrations), this may happen anywhere along the intestinal stretch. Calcium diffusion is greatest in the intestine (Ross *et al.,* 2011).

2.3.1.3. Calcium Homeostatic Regulation

Endocrine system plays a very important role in maintaining a narrow physiological range of circulating ionized calcium. Calcitriol's hormonal activities are intimately related to calcium balance in the body (Ross *et al.,* 2011). In mammals, the calcium homeostatic process is based on the vitamin D metabolic system (Fig. 2.11). Total calcium levels in the blood are closely regulated in the range of 8.5 - 10.5 mg/dL (2.12 - 2.62 mmol/L) (Jeon, 2008). If this amount decreases significantly, the parathyroid gland's calcium sensing receptor, a calcium sensor signals the secretion of parathyroid hormone. The kidney is stimulated to produce calcitriol, a source of vitamin D, which is to activate bone resorption, this raises the extracellular calcium concentration levels.

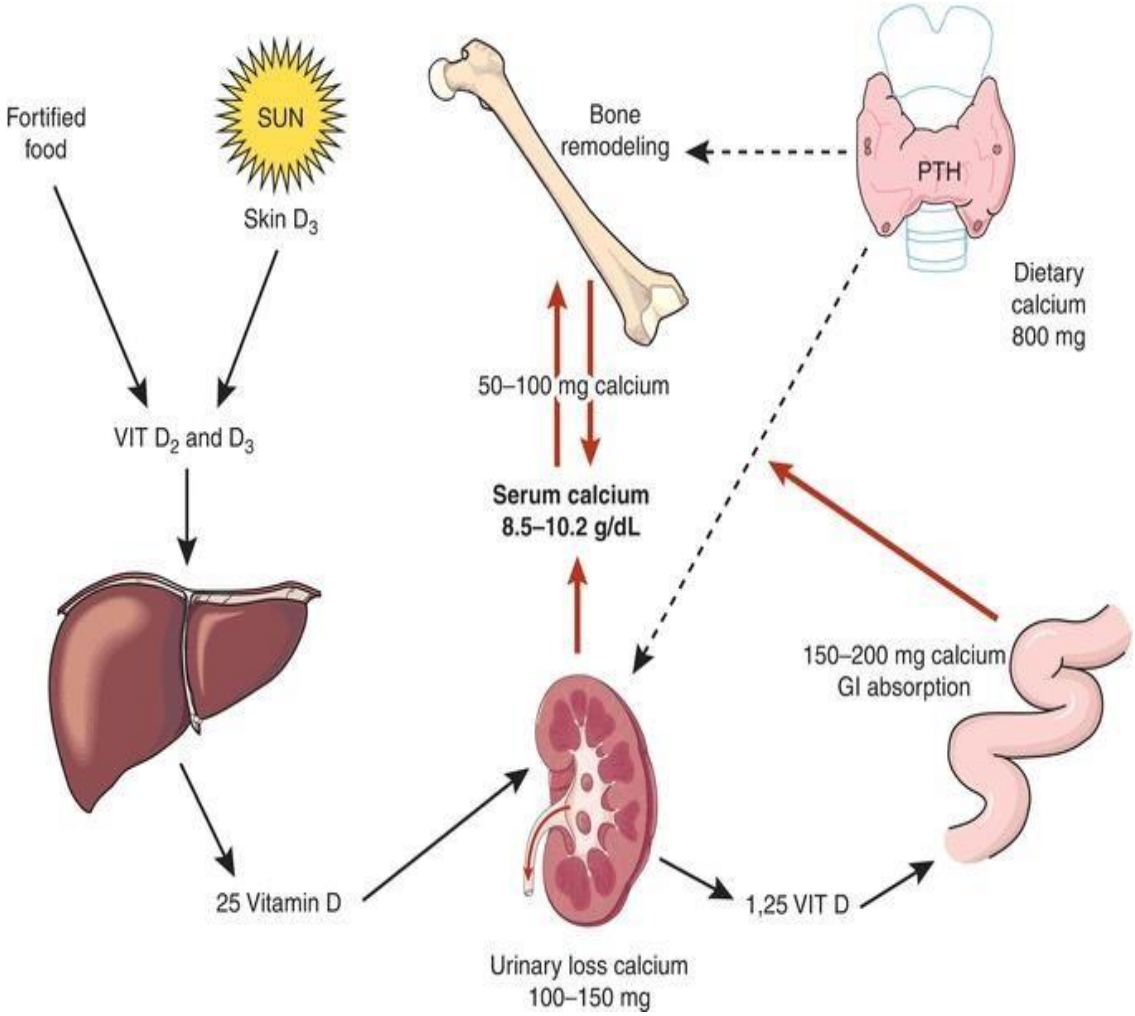


Figure 2.11: Organ system integration of calcium homeostasis (basic medical key, 2017).

Calcitriol also raises serum calcium level by acting on the intestine, bone, and kidney as hormone. It also raises phosphate level by acting on the intestine, and to a lesser degree, on kidneys. As serum calcium level increases, the calcium sensing receptor is shut off, and PTH secretion decreases. When blood calcium level is elevated, the thyroid glands C-cells (parafollicular) produce calcitonin, which prevent bone calcium resorption and maintain normal serum calcium levels. Calcitriol also provides feedback by blocking the synthesis and release of PTH via its receptor, a process known as PTH suppression. Serum phosphorus levels also influence calcitriol synthesis; a high level inhibits calcitriol production, but a reduced level promotes its production.

2.3.1.4. Excretion

Calcium is mostly excreted in urine and feces, although Ca is also available in many other fluids of the body, including sweat. Urine Ca is determined by the ratio of calcium content filtered by the renal glomeruli to the ability of reabsorption from the renal tubules (Hoenderop *et al*., 2000). Approximately 92 percent of filtered calcium is reabsorbed at four different areas of the kidney through active or passive processes, both of which help to keep the calcium balance neutral. The sensing receptor for calcium available in the ascending loop of Henle responds to extracellular fluid high calcium concentrations by regulating active calcium transport by blocking active reabsorption in the loop, allowing70% of filtered calcium to be passively absorbed into the proximal tubule (Ross *et al.,* 2011). Low filtered calcium levels stimulate calcium sensing receptor to completely reabsorb filtered calcium. TRPV5 ion channels (transient receptor potential cation channels) regulate active calcium transport in the distal tubule through calcitriol and estradiol (Hoenderop *et al.,* 2000). Finally, a small amount of total calcium is reabsorbed in the collecting tubule, it may be involved in calcium transport in the passive state (Ross *et al.,* 2011). Renal excretion removes about 5 mmol of calcium per day from a healthy adult (Weaver & Peacock, 2011).

Intestinal unabsorbed calcium is excreted in feces, while unabsorbed Ca in the mucosal cells secretions is excreted as saliva, bile, gastric juices and pancreatic juice. Endogenous calcium excretion refers to the loss of calcium by the intestines, as well as small losses by sweat. In comparison to urinary excretion, endogenous calcium excretion is not altered significantly by age (Ross *et al*., 2011). PTH determines urinary calcium excretion; once calcium intake decreases, it causes increase in PTH levels which in turn

decrease excretion of urinary calcium. Age-related impairment of renal function impaired filtration and decreases calcium loss, it also causes a rise in levels PTH secondary to decreased phosphate clearance. Despite the fact that calcium is actively reabsorbed in the kidneys, impaired renal function, which may result in reduced active transport of calcium, could result in calcium net loss from kidneys.

2.3.1.5. Excess Calcium Intake

Excess calcium intake is often not caused by calcium intake through foods, but excessive calcium supplementation has been recognized as the main cause of hypercalcaemia (Beto, 2015; Cormick & Belizán, 2019). Excess calcium intake may have adverse implications, the most common of which is the formation of kidney stones (Ross *et al.,* 2011). Calcium interacts with a number of other elements and is involved in the metabolism of many of the cells of the body; hence, disruptions in calcium metabolism can have a range of negative consequences (Ross *et al.,* 2011).

2.3.1.6. Calcium and Autism Spectrum Disorders

Regulation of calcium has been linked to synaptic plasticity and neurodevelopment. It is in charge of transmitting electrical signals along nerves, thereafter, changes in calcium levels can result in abnormal signal transmission along nerves and developing neurons. (Lohmann,2009; Sadakata & Furuichi, 2010; Muldoon *et al.*, 2015). The extracellular calcium-sensing receptor (CASR), which is responsive to extracellular calcium levels, detects calcium levels in the parathyroid gland. The receptor is also located on developing neurons and aids neurite outgrowth regulation in the postnatal brain (Liu *et al,* 2013). Thus, severe or sporadic hypocalcemia in ASD individuals can cause a deleterious impact in synaptogenesis and neuron outgrowth; this can impair neurodevelopment, which may eventually manifest as neuropsychiatric complications in these individuals (Muldoon *et al.,* 2015). The mechanism is unknown but there are accumulating evidences that there is a calcium dysregulation in ASD (Nguyen *et al*., 2018; Rylaarsdam & Guemez- Gamboa, 2019). Dysregulation is a term used to describe a state when both environmental and genetic influences are considered, and calcium is confirmed to be the most prominent biological risk factor of ASD (Napolioni, 2011; Zeidán-Chuliá *et al.,* 2013; Emberti Gialloreti *et al*., 2019). Calcium homeostatic mechanisms have now been associated with pathogenesis of ASD and other neurological conditions (Harrison, 2015; Nguyen *et al*., 2018).

2.3.2. Magnesium (Mg)

Magnesium is the eighth most abundant mineral in the Earth's crust, the body's fourth most available cation and the second largest intracellular cation (Luft, 2012; Jahnen-Dechent & Ketteler, 2012). Like calcium, magnesium is a member of Group II element on electrochemical series table. Mg has a valency of +2; hence, it is involved in electron donation in most of its biochemical reactions. It is essential for physiological role in a number of bodily functions (de Baaij *et al*., 2015). Magnesium has a number of functions, including chelating important inorganic anionic ligands, particularly ATP, as well as competing against calcium over binding sites on protein and membranes (Ryan, 1991). Magnesium assists in the maintenance of intracellular free calcium concentration by trying to compete for membrane binding sites with calcium ion as well as enhancing calcium sequestration via sarcoplasmic reticulum.It also plays some crucial roles in a range of biological activities like cell replication, cellular energy metabolism and protein synthesis (Noronha & Matuschak, 2002; Seo &Park, 2008). Magnesium is required by over 300 enzymes for metabolic activities.

2.3.2.1. Sources

Magnesium, just like calcium, is essentially derived from the diet. Magnesium is influenced by magnesium concentrations in drinking water magnesium and food compositions (Nerbrand *et al.,* 2003). Drinking water, especially hard water, is an important source of magnesium (Sengupta, 2013). Mg level is abundant in green leafy vegetables, legumes, nuts, cereals and grains, moderate in chocolate, non-green vegetables, poultry and fish, but low in dairy products. Magnesium consumption is typically directly proportional to energy intake unless the main energy comes from alcohol or a refined sugar (Seo & Park, 2008). Magnesium content of food can be reduced by up to 85% when it is refined or processed.Cooking/boiling magnesium-rich foods results in substantial Mg loss (Rude, 1993). Therefore, that many populations seem to have a high incidence of low magnesium intake may be explained by food processing and cooking.

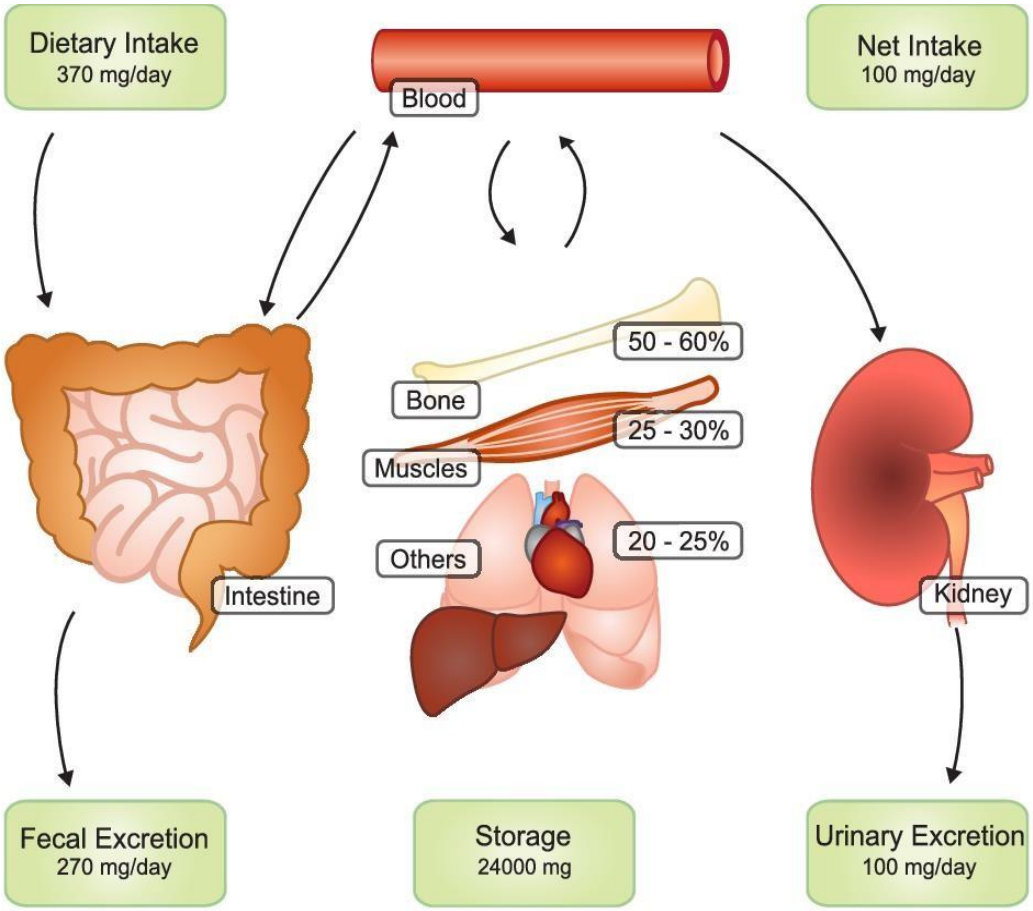
2.3.2.2. Metabolism

The metabolism and activities of magnesium is closely related to that of calcium. Bone stores about 60% of the body's total magnesium, 30% of which is exchangeable and serves as a buffer to keep plasma concentrations stable. In regards to intracellular

magnesium, skeletal muscle stores about 20% of total body magnesium, other soft tissues store 19% and extracellular fluid stores less than 1% of total body magnesium (Seo & Park. 2008). Total serum magnesium level in healthy adults ranges from 0.70 to 1.10 mmol/L. About 20 percent Mg are bound to protein, while almost 65 percent are ionized, and the remaining percentage is complexed with anion of phosphate and citrate (Saris *et al.*, 2000; Seo & Park. 2008). Albumin is responsible for 60–70% of the protein bound fraction, while globulins are responsible for the remainder (Kroll & Elin, 1985). Homeostatic control of plasma Mg level is largely done by the kidneys. Normally, about 80% of total body magnesium is filtered, out of which 95% is reabsorbed, leaving just about 5% to pass through the urine (Fig. 2.12).

2.3.2.3. Metabolism in Neuromuscular and Central Nervous System Activities

Several works have reported low dietary intake of Mg in clinically diagnosed autistic spectrum disorders (Omotosho *et al*., 2017). Magnesium deficit can manifest in various of ways, including hypocalcaemia, hypokalaemia, cardiac, and neurological symptoms (Swaminathan, 2003; Mehta *et al.*, 2016). Neuromuscular and neuropsychiatric disturbances are typically the first signs of magnesium deficiency. The wide range of neuromuscular problems caused by magnesium deficiency could be due to a number of mechanisms, including axon instability (Kirkland et al., 2018). When serum magnesium levels are low, the axon stimulation threshold is lower and nerve conductivity velocity is higher. Magnesium influences neurotransmitter release at the neuromuscular junction and triggers hyper-responsive neuromuscular activity by competitively inhibiting calcium entry into presynaptic nerve terminals (Omar, et al., 2020). Magnesium has an effect on calcium processing in muscle cells, which affects muscle contraction and relaxation (Potter et al., 1981). Magnesium deficiency increases calcium release from the sarcoplasmic reticulum. Furthermore, magnesium is also necessary for calcium re-uptake. A low intracellular magnesium concentration has the net effect of increasing muscle contraction to a givenstimulus while reducing the muscle relaxation ability, thus rendering the muscle impervious to tetany (Swaminathan, 2003).



RE 2. Magnesium homeostasis. Panels represent the daily amount of Mg^{2+} intake and excretion

Figure 2.12: Magnesium Homeostasis (Jeroen *et al.*, 2015)

2.3.2.4. Distribution in the Body

The jejunum and ileum are the primary sites of magnesium absorption where approximately 30-40% of the magnesium in the diet is absorbed. The fractional absorption is inversely related to intake, i.e., it is high (65 percent) when the intake is low, low (11 percent) when the intake is high and normal when intake is passive (Kayne & Lee, 1993). Although the factors that regulate magnesium absorption are unknown, studies indicate that parathyroid hormone (PTH) can be involved in the process. The role of vitamin D as well as that of its active metabolite 1,25 dihydroxycalcelciferol in Mg absorption is unknown (Swaminathan, 2003). Aside from these, some dietary factors have been postulated to affect magnesium absorption. These includes presence of oxalate, phosphate, proteins, potassium and zinc (Seo & Park, 2008).

The effects of magnesium deficiency on the nervous system are complicated and poorly understood. Magnesium deficiency causes intracellular calcium accumulation and subcellular calcium transport disturbances. Magnesium deficit is related to excitatory neurotransmitters like serotonin and acetylcholine, blockage of the N- Methyl-D-aspartate receptor non-competitive role and most probably, the inhibition of amino acid γ-amino butyric acid's inhibition ability (Swaminathan, 2003).

2.3.2.5. Magnesium and Autism Spectrum Disorders

Autism spectrum disorders have all being linked to magnesium metabolism (Mousain-Bosc et al., 2011). Magnesium has been said to prevent developmental delay in children. Mg is reported to show significantly lower Erc-Mg values in ASD than controls (Mousain-Bosc *et al*., 2011; Omotosho *et al*., 2018). Traumatic brain injury has been associated to a reduction in Mg^{2+} concentrations in the bloodstream, which can lead to neurologic deficits (Vink *et al.,* 2009). Many studies also observed that Mg has ameliorative effect when used in combination with Vitamin B6 in the management of ASD. Mg-vit. B6 regimen have been administered in a treatment regimen, which leads to an increase in Erc-Mg values with no side effects; the supplementation improved autism symptoms in the larger percentage of children (Mousain-Bosc *et al*., 2006).

The neurobiological rationale for Mg/vit B6 supplementation was its possible reversal of ASD's damaged neuronal Mg pathway. Mg regulates ion transfer across membrane channels and functions as an ionic membrane regulator. Magnesium salt has also been used to treat brain ischaemia, a disorder that induces a decrease in free intracellular

Mg^{2+} levels (Xue *et al*., 2020). Administration of magnesium salts in this condition has been found to improves motor outcome (Ebel & Gunther, 2005). Mg has been linked to improvements in release of neurotransmitters, excitability of the neurons, and other types of synaptic plasticity such as lengthy synaptic transmission impairment in older people, according to a review article by Billard (2006). In research animal, an improvement in brain magnesium improves learning capacity, working, short and lengthy memories (Slutsky*et al*., 2010). This means that increasing Mg in the brain helps with short and long terms synaptic facilitation. Learning and memory are also aided by the supplement. Magnesium influences neurotransmitter activation as well as other modulators and mediators. (Hoane, 2011). Magnesium acts as an agonist (turns on) for GABA activation receptor, an inhibitory pathway in the nervous system, which in some cases in ASD may lead to increased behaviour. Magnesium also plays an antagonist role for NMDA glutamate receptors, the excitatory pathway in the nervous system which can have effect in neuroprotection and thus protect against autistic regression episodes (Mousain-Bosc et al., 2011). There is evidence of an irregular inhibitory–excitatory balance in ASD brain, which could be targeted by therapy by affecting GABA and glutamate neurotransmission (Nelson & Valakh, 2015). In summary, variations in Mg level have been reported to affect neurotransmission by modulating the GABA and glutamate neuronal transmission.

2.3.3. Lead (Pb)

Lead (Pb) is a member of Group IV element and its biochemical reactivity is based on number of electrons on its outer shell that is available for donation during most biochemical processes. This makes it possible for Pb to replace elements like Ca and Mg in many metabolic processes including the enzymatic reactions where they function as cofactors. Pb is an environmental toxic element that has essential properties like poor conductivity, softness, corrosion resistance and malleability, making it difficult to be fully removed (Wani *et al.,* 2015). Non-biodegradable nature and long-term use of Pb cause its builds up and pose a growing danger. Pb is recognized as one of the major risk indicators of many diseases that affect human beings (WHO, 2000). Despite the fact that its use has been phased out in many countries, it remains indispensable material among some industries, such as auto repair, battery manufacturing, recycling, mining, and smelting (CDC, 2012). African children generally have been reported to be vulnerable to Pb exposure and toxicity due to continuous use of leaded gasoline and lead-acid batteries in automobiles (Omokhodion, 1994). Also, children exposure to elevated level

of Pb is attributed to paints and household dust (Ogunseitan and Smith, 2007). In Nigeria and other African countries, gasoline has been reported to contain 0.5-0.8g/L Pb, the highest levels of Pb in gasoline globally. Nigeria gasoline in particular contains 0.66g/L lead (Fakayode & Olu-Owolabi, 2003).

2.3.3.1. Sources of Exposure

Man is exposed to lead and its compounds primarily through lead-related occupations, which include leaded petroleum, lead smelting, use of lead-based painting, combustion, lead-containing tubing, lead battery charging and recycling (Fig. 2.13) (Sharma & Dubey, 2005). Due to children increased rate of absorption of ingested lead in children, as well as their inherent curiosity and age-appropriate hand-to-mouth behaviour, they are especially susceptible to lead poisoning. This results in mouthing and swallowing of lead-containing objects, and the major identified sources include house paints, leaded pencils and toys (Thurmer *et al.*, 2002). The vulnerability of growing children to Pb toxicity is anchored on their developing organs, particularly brain; which makes children more susceptible to neurodevelopmental and immunological disorders, among others.

Other sources of exposure: Apart from normal environmental exposure by food and drink, individuals can be exposed to lead by habit and unavoidable circumstances. These other exposures may be classified as being caused by atmospheric lead or unrelated to environmental lead. The first to be considered is dust that contains high level of lead and is available in many residential areas, refineries and high-density traffic. Individuals may obtain it through inhalation and consumption of fruits and vegetables planted near lead emissions plant or on lead soils. Secondly, exposure to lead is also possible through family members occupationally exposed to lead and also through contamination of house dust, use of leaded paint, contamination of water in homes with lead water, cigarette smoking (Ewers et al., 1990) and alcohol (especially wine) consumption (Elinder et al., 1983).

2.3.2.2. Routes of Exposure to Lead

Lead present in both indoors and outdoors dust is a major source of ingestion by children, especially in a polluted environment (Ewers *et al.*, 1985).

Inhalation: The majority of Pb in the environment is sub-micron-sized particles. The respiratory system retains 30–50% of the particles inhaled. The body absorbs almost all

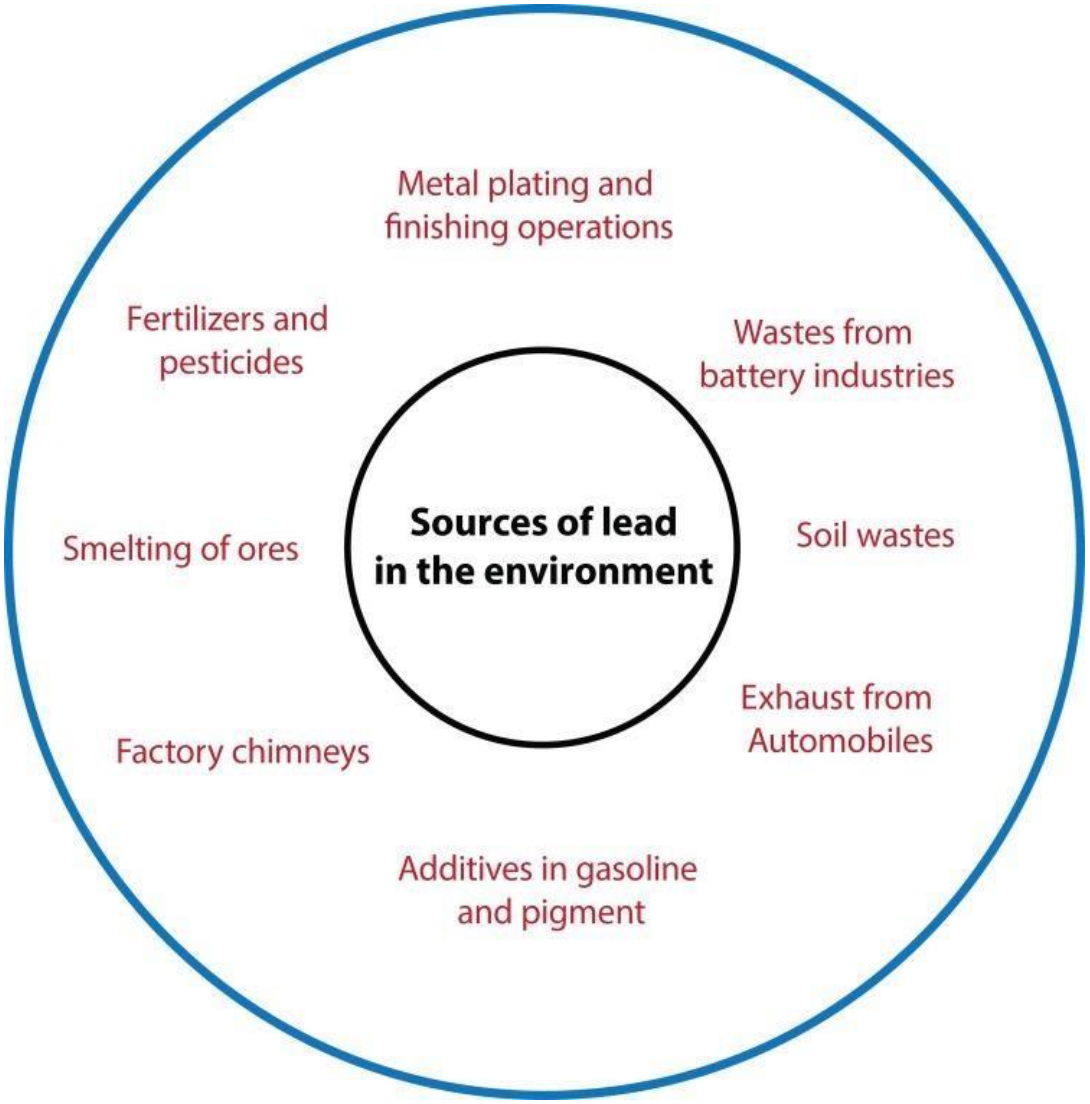


Figure 2.13: Environmental lead sources (Sharma & Dubey, 2005)

of the lead that has been retained. Lungs effectively often stored particles with a diameter of 1–3m. Larger particles are deposited with varying degrees of effectiveness in the upper respiratory tract, where absorption is not completed. Lead particles cleared by the lungs may be swallowed; this cause more lead to be absorbed in the intestinal tract (Ewers et al., 1985). Metrological and physical characteristics have significant effects on the amount of environmental Pb. When wind velocity is high, particles are transported quite quickly (Thomas, 2013).

Ingestion: Drinking water and ground water contain Pb levels ranging from 1 to 60g/l. Lead levels in domestic tap water in most European countries are relatively low, usually 20g/l. As a result, lead exposure from water is relatively small when compared to food lead exposure. In a study done by Kramer and others, it was reported that blood Pb level reported in children living in houses which has lead pipes for drinking water was 30 percent higher than those living in houses without lead pipes (Krämer *et al.*, 1994). Lead in drinking water may be higher and contribute largely to the total lead intake, particularly in areas where lead pipes are prevalent (Payne *et al.*, 2008).

Food: The majority of people get Pb from food on daily basis. Majority of foods, including beverages and alcoholic drinks stored in cans, are contaminated with Pb in the process of manufacturing and storage (Kregiel, 2015; WHO, 2022). Direct foliar contamination of plants is considered to be the most critical source for atmospheric lead to gain access to the foodchain (Kumar *et al.*, 2020). This contamination is determined by the rate of lead fallout in the agricultural districts; it is increased in highly industrialized places. Furthermore, air droplets increase amount of lead in soil; this could increase lead absorption from the roots over the course of decades or centuries. The levels of lead in different foods are extremely variable. Adults' average lead intakes have been recorded to be in the range of about 100– 500µg/24hrs in many studies, with food contributing the larger amount; according to a report, daily total intakes are around 100µg or less. Estimates of overall daily intake for young children are about one-half of those for adults. (Pennington and Schoe, 1995).

Other routes of exposure: Although dermal exposure is a factor in worker exposure to organic lead, it is not thought to be a significant pathway for the general public. Through the skin, organic lead may be immediately absorbed. Tetraethyl lead, an organic form of lead, is more likely than inorganic lead to be absorbed through the skin. Those who

work with lead or items that contain lead are more likely to experience dermal exposure (ATSDR, 2017).

An individual's current BLL may be dramatically influenced by endogenous lead exposure. A history of military service or other penetrating injuries may be crucial, as numerous studies describe lead poisoning brought on by retained bullet or shrapnel pieces (Kathuria, 2014). This presents a particular risk to the growing foetus if in a pregnant woman. If the mother is exposed to lead, there may be trans-placental exposure to the foetus. Lead can be kept in mineralizing tissue for a very long time after it has been taken into the body (e.g., teeth and bones). When the body is under calcium stress (such as during pregnancy, lactation, or osteoporosis) or when there is a calcium deficiency, the stored lead may be released back into the bloodstream (ATSDR, 2017).

2.3.3.3. Metabolism

Ingestion, inhalation and skin remain route of lead (Pb) absorption into the body (ATSDR 2010). The major route of absorption in most individuals is through the GIT (ATSDR 2010). The particle size distribution and ventilation rate have an influence on absorption through the respiratory tract. The retention rates of airborne particulates in adults vary between 20% and 60%. Although the chemical form of lead varies greatly in terms of water solubility, it is not known to be a significant factor in respiratory absorption (Wani *et al.*, 2015). In adults, gastrointestinal tract absorbs about 10% lead, whereas in children, levels as high 40% - 50% are absorbed (Kiela & Ghishan, 2016). Conlon and Bird (2014) found that dietary or nutritional factors/status have a significant impact on gastrointestinal absorption: it is improved by milk and fasting. Certain substances, like Ca, Fe, Mg and fat can reduce lead absorption,while low zinc and vitamin C may increase lead uptake (Zhai *et al.*, 2015). Lead absorption and bioavailability in the GIT is also dependent on its salt water solubility (Cheng & Wong, 2020). This must be taken into account when estimating direct lead intake by children from soil, as well as when measuring pH of the gastrointestinal tract (ATSDR, 2019).

2.3.3.4. Distribution

Blood, soft tissues, and mineralizing tissues all contain the non-excreted fraction of absorbed lead (bones and teeth). In blood: adults have about 95 percent of their lead body burden in the bones, compared to about 70 percent in infants (Barry, 1981). Only

a fraction of one percent of the lead in the bloodstream is in plasma, while 99% remains bound to erythrocytes (red cells). As blood lead levels rises, plasma lead fraction also rises, resulting in a curvilinear relationship between the two. Pb concentrations in bones increased with age, and this rise is more pronounced in males' denser tibia bones (WHO, 2001).

The distribution of the absorbed lead when it reaches highest concentration is from blood to the soft tissues, such as the liver, the kidney and the brain (Andrén *et al*., 1998). Lead is present as intra nuclear inclusion bodies in these organs of the body. Lead does cross the blood-brain barrier to some level (Rădulescu & Lundgren, 2019). Grandjean (1975) reported that lead is not evenly distributed in the nervous system; however, hippocampus, amygdala, as well as the choroids plexus have increased lead levels (Manton & Cook, 1984). Lead entry into the nervous system is somewhat greater in infants and young individuals compared to adults. The amount of lead levels in cerebrospinal fluid is extremely low (Sanders *et al*., 2009; Skerfving & Bergdahl, 2015).

2.3.3.5. Elimination

Almost 76% of the absorbed lead is mainly excreted though urine while fecal excretion accounts for 16% and the remaining 8% of the absorbed lead is excreted through sweat, hair, nails as well as other body tissues (Christensen & Kristiansen, 1994). Non-absorbed lead on the other hand is excreted in the faeces after passing through the gastrointestinal tract. Surprisingly, little information exists on lead retention and excretion as a function of age. Accordingto some findings, infants were reported to retains highest levels of lead (Kiela & Ghishan, 2016).

2.3.3.6. Health Effects

Lead toxicity is primarily due to its interaction with various enzyme systems; lead binds to protein SH-groups or displaces other important metal ions and inactivates these enzymes (Sanders *et al.,* 2009). As a result of this, lead has since been related to a variety of biological effectand many organs and structures are possible targets for lead (Flora *et al.,* 2011). Among the systems affected by lead effects are haem biosynthesis, central nervous system, renal system as well as the reproductive system, among others. In addition, effect of Pb on the cardiovascular, hepatic, endocrine and gastrointestinal systems have been reported (Flora *et al.,* 2011). In circumstances of reduced level but long-time lead exposure, like those observed in the general public and occupational

environments, the effects on the nervous system, haem biosynthesis, the kidney and erythropoiesis are the most critical (NRC, 1993; WHO, 2022). The effect of Pb on haem biosynthesis and the nervous system is one of the most researched aspects of the element. Several markers were identified in many experimental studies on Pb. However, inhibition of δ-aminolevulinic acid dehydratase (δ-ALAD) activity—a rate limiting enzymes in biosynthesis of haem—is among the earliest lead toxicity biomarkers. Comparatively, experimental evidence has shown that the brain of a developing young rodent is significantly vulnerable than that of developed adult rodent (WHO, 2001).

2.3.3.6.1. Effects on the Nervous System

Plasma flows into the interstitial spaces of the brain when the blood-brain barrier is exposed to high levels of lead, causing edema. When the CNS is exposed to high levels of lead in the blood, it develops encephalopathy and edema, which mostly damages the brain's cerebellum (Fig. 2.14). Edema creates dangerously high levels of pressure in the brain, which can result in irreparable damage. Reduced attention, visual motor reasoning skills, social conduct, and mathematic and reading abilities are all symptoms of this type of brain damage ((Davis & Svendsgaard, 1990). According to studies, lead poisoning reduces cognitive function by 0 to 5 IQ points for every 10 g/dl increase in blood lead levels (Brochin *et al*., 2008). Toxicity of lead has been demonstrated experimentally on both young and adult animals,as previously described. Pb toxicity has been attributed to encephalopathy in humans (Wani et al., 2015). Adults with more than 1200 g/l and children with 800 and 1000 g/l blood lead levels have also been diagnosed with encephalopathy.

Pb toxicity of the nervous system has observed clinically in a variety of ways. In infants, the effect is always fatal, and many who do recover often suffer from permanent neurological and neurocognitive consequences. (Davis & Svendsgaard, 1990). Alternatively, experimental research into the nervous system has been conducted, with neuro-behavioural functions studied in both lead workers and children exposed to Pb environmentally (Sanders *et al.,* 2009). Many studies have discovered that lead employees perform worse on cognitive and sensorimotor tests than controls (Saddik *et al.,* 2005). In lead-related studies of neuro-behavioural deficits at environmental lead level exposure, children have received special attention as a risk group for CNS effects.

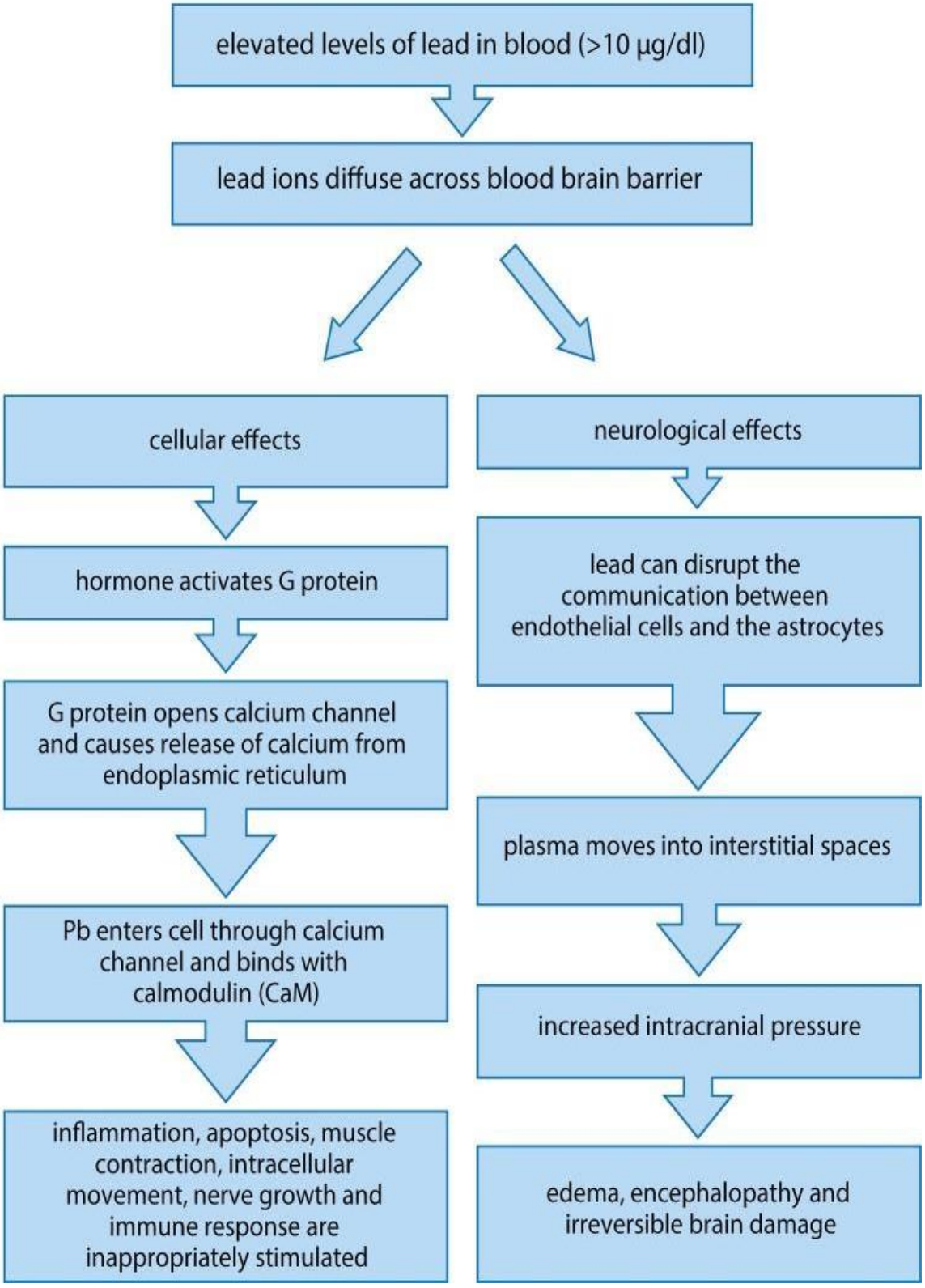


Figure 2.14: Increased Blood Lead Level Health Effects (Brochin *et al.*, 2008)

Blood lead levels below 300µg/l have been reported in both cross-sectional and prospective trials. Apart from a variety of neuropsychological tests that cover aspects of attention and visual motor performance, cognition have gotten special attention as a world indicator of nervous system functionality. It was reported that increasing PbB levels from 100 to 200µg/l is correlated with a 1–3-point decrease in IQ (Chwartz, 1994; WHO, 1995), the social consequences of which may be significant if population-shift hypothesis is correct (Needleman *et al.,* 1982). Despite all of the observations, there is no conclusive proof of a threshold in the existing epidemiological studies. The presence of such a deficit in adults has been reported with PbBs of 500 g/l as compared to controls of 100 g/l (Sanders *et al*., 2009).

2.3.3.6.2. Effects on Pregnancy and Offspring

The toxic effect of lead on pregnancy and newborns *in-utero* has been severally reported. Figure 2.15 depicts the different routes by which infants can become exposed to lead. Haemodilution, which is a major phenomenon in pregnancy, increases the blood volume,causing an initial decrease in blood-Pb level (Schnaas *et al.,* 2016). Pb is mobilized from skeleton in those occupationally exposed like a feedback mechanism, causing a rise in blood Pb level (Téllez-Rojo *et al.,* 2004). This increase has been reported to be up to 20% in an Australian study of women, with 30% of that coming from the skeleton (Gulson *et al.,* 1998). Some reports have also indicated a relationship between calcium and Pb in Pb exposure. Reduced calcium intake is linked with an increased skeleton lead mobilization, especially in cases of a higher exposure to Pb (Rădulescu & Lundgren, 2019). Generally, increase in blood Pb as seen in occupational Pb exposure has been attributed to hypertension in pregnancy, particularly in women occupationally exposed to Pb. In the placenta, lead accumulates (Wani *et al*., 2015).

One of the major concerns about Pb exposure in pregnancy is its ability to cross placental barrier into the circulation of developing fetus (Fig. 2.16). Lead in mother's blood enters foetus circulation through the placenta and umbilical cord by passive transport. Lead ions infiltrate the blood brain barrier leading to cognitive impairment. Several studies have confirmed deposition of Pb in placental of occupational-exposed pregnant women (Esteban-Vasallo *et al*., 2012). Amniotic fluid contains low amounts of lead; however, the placenta transfers a significant amount of skeleton-mobilized and GIT-absorbed Pb into the developing fetus (RÍsovÁ, 2019). The maternal and cord B-Pb concentrations

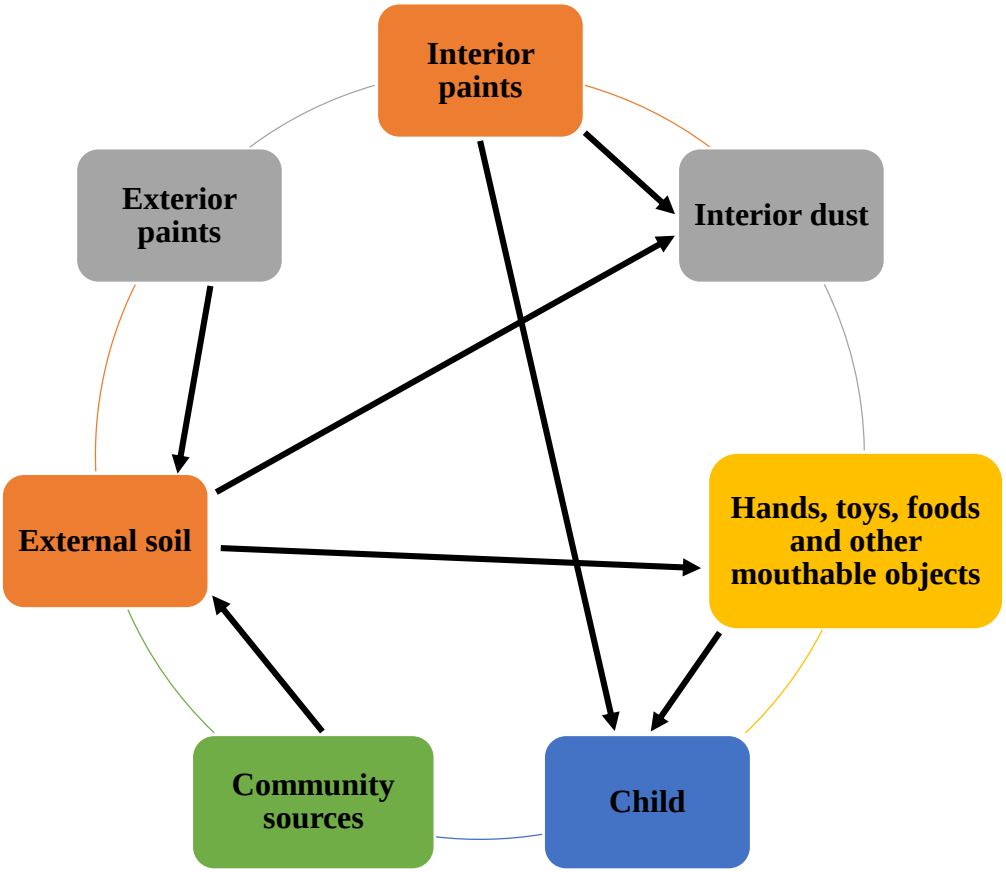


Figure 2.15: Scheme showing sources of children exposure to Lead in the environment (Dobrakowski *et al.*, 2014).

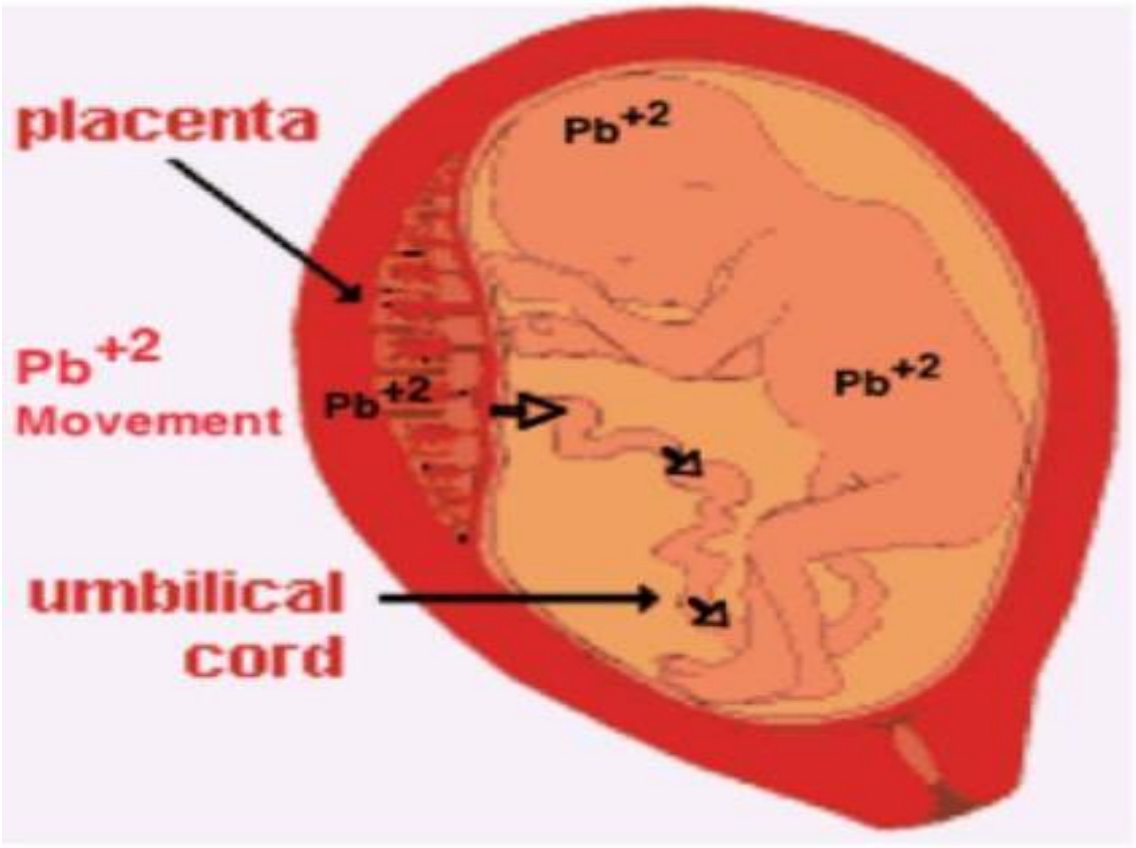


Figure 2.16: L ead movement from maternal blood to fetus circulation (Brochin *et al.*, 2008)

are nearly identical; approximately 85 percent of the maternal Pb concentration was reported in cord blood (García-Esquinaset al., 2013). In laboratory animals, lead is toxic to both embryo and foetus (RÍsovÁ, 2019). Lead exposure in pregnant women has been linked to spontaneous abortion on many occasions (Amadi *et al*., 2017). However, most studies were skewed due to limited number of samples, inadequate result ascertainment, lack of confounding control, and/or exposure evaluation flaws. Even when these variables were taken into consideration, there was still a correlation in risk factors and blood lead level (Nakhaee *et al.,* 2019). Thus, the sensitivity to lead exposure differs greatly between individuals; although the mechanism remains unclear, epigenetics appears to be involved (Baroukiet al., 2018).

2.3.3.7. Toxicity

Consumption of food and water contaminated with lead remain the common cause of Pb poisoning. Inhalation of dust and leaded paint can also be accounted for poisoning. Ingested lead absorbed into body through GIT has deleterious effect on body organs and systems. It has a great impact on kidneys, nervous systems and immunity (CDC, 2014). Nervous system is the most affected in children and adults, although the children suffered greater impact because they are undergoing rapid growth and development (Bas et al., 2015). Infant's brain is sensitive, even to low Pb exposure, and may cause developmental and learning delay (Bas *et al*., 2015). Lead toxicity is dangerous and harmful; it can cause permanent health challenges (WHO, 2021).

The mechanism by which lead toxicity is inflicted has been extensively studied at cellular and sub-cellular levels. In many of these studies, toxicity of Pb in cells has been largely attributed to oxidative stress and ionic displacement (Fig. 2.17), with the consequent effect on enzymatic activities and metabolic processes (Aliyu & Amanabo, 2021). Oxidative stress causes health damage as a result of high rate of production of free radical and reduced body ability to detoxify the free radical (Pizzino *et al.,* 2017). Lead increases free radical production and reduce antioxidant reserves that have been previously produced (Flora *et al*., 2012). Lead has electron-sharing capacity and attached to the sulfhydryl groups contained in antioxidant enzymes and rendered them inactive. The antioxidant enzyme glutathione gets inactivated in this way, resulting in synthesis of GSH from cysteine (Forman *et al*., 2009). Also, other enzymes that reduce glutathione levels such as glutathione reductase (GR), glutathione peroxidase (GPx), δ-

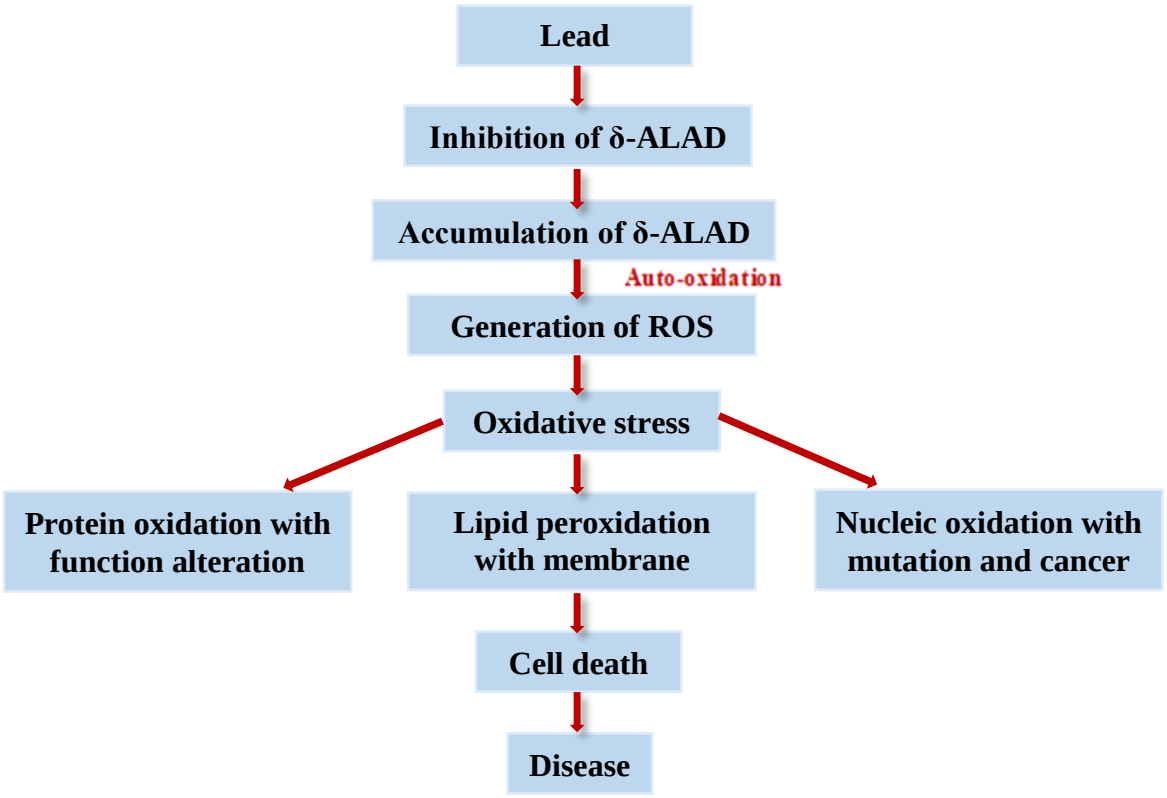


Figure 2.17: Mechanism for lead-induced oxidative stress and cell death (Sani, Aliyu & Musa, Amanabo, 2021).

amino levulinic acid dehydratase (ALAD) and Glutathione-S-transferase are all inactivated by Pb (Flora *et al*., 2012). One of the mechanisms of toxic effect of lead is the ability to replace bivalent cations including Ca^{2+} and Mg^{2+} that have important physiological and functional roles within the body (Wani *et al*., 2015).

Apoptosis, enzyme control, ionic transportation, neurotransmitter release, and other fundamental cellular processes are all affected by Pb (Garza *et al.*, 2006). This process is largely responsible for cognitive deficits, as Pb at an appreciable level cross the blood-brain barrier (BBB) and displaces calcium ions. Lead accumulates in astroglial cells after passing the BBB. Lead is more toxic to the immature astroglial cells of the developing nervous system. It damages immature astroglial cells easily and prevents myelin sheath formation which is very important in BBB development (Lidsky & Schneider, 2003). Lead may replace calcium, even at very low concentrations affecting main neurotransmitters excitation effect and memory storage (Flora et al., 2012). It also affects concentration of sodium ion in a number of important biological functions like cell-to-cell communication, neurotransmitters absorption and synaptosome calcium uptake and absorption (Flora *et al*., 2012).

2.3.3.7.1. Lead and Neurodevelopmental Disorders (ASD and CP)

Environmental lead (Pb) and manganese (Mn) levels have been linked to negative impact on neurodevelopment (Neal & Guilarte, 2013; Lucchini *et al*., 2017). Lead is a possible risk factor in development of NDDS, as blood lead concentration was reportedly elevated in ASD and CP compared to neurotypical children (Zafeiriou *et al.*, 2013; Omotosho *et al*., 2018; Akinade *et al*., 2019). It cannot be ascertained if elevated lead level is the main cause or consequence of autism because autistic children have a typical feeding habits, such as habitual mouthing and pica. Blood lead levels are also reported to be higher in CP cases, which may exacerbate cognitive decline and neurobehavioral issues (Bansal *et al.*, 2017). Early-life lead exposure can result in permanent brain damage including CP (Bansal *et al.*, 2017).

2.3.3.7.2. Lead Toxicity and Autism Spectrum Disorders

Many researchers found increased blood lead levels in people with ASD, indicating lead toxicity (Lidsky, 2005; George *et al.*, 2010). In retrospective uncontrolled research, Cmambel *et al*. (1980) found lead level above 35g/dl in 19% of the study population and

that blood lead level has indirect relationship with cognitive functioning. In an ASD and environmental toxicant study, blood lead level was seen to be higher in ASD (Rossignol *et al.*, 2014).

Many researchers reported elevated blood lead levels in people with ASD, although four case-control studies found no evidence of increased blood lead levels in individuals with ASD controls (Rossignol et al., 2014). In a study done in Saudi Arabia, it was reported that 14 ASD children had significant higher blood lead levels than 12 age-matched controls (Rossignol *et al*., 2014). Another case-control analysis from Saudi Arabia reported that 25 ASD childrenhad significantly increased blood lead levels than 16 age-matched controls (Blaurock-Busch *et al*., 2011). Cohen & colleagues (1982) reported a variation in lead level in ASD, Tourette syndrome children and neurotypical children. However, case-control analysis found similar blood lead concentrations in ASD and age-matched control (Cohen *et al.,* 1982). In addition, same result pattern was reported by Albizzati and his colleagues is a study done on whole blood of 17 ASD and 20 TD children (Albizzati *et al.*, 2012) as well as plasma of 28 ASD children and 32 TD (Albizzati *et al.,* 2012). Generally, there have been several reports for and against a direct implication of elevated Pb in neurodevelopmental disorders. However, the preponderance of evidence supports adverse effect of elevated Pb level on neurodevelopment, especially in the growing child.

2.3.4. Aluminium (Al)

Aluminium (Al) is one of the metals most commonly used in homes and environment. This is a flexible metal with a range of properties and applications. Aluminium is indeed a toxic metal that serves no purpose in the human or animal body, and its toxicity is accessed vis routes of exposure and the solubility of aluminium compounds (Davidson *et al*., 2007). Aluminium can get into human body through food, drinking water, fruit juices, wine and beer, or through everyday items made of aluminium, such as cosmetics and pharmaceuticals. Aluminium is a documented neurotoxic substance that accumulates in the brain after being ingested. The building-up of aluminium in brain can cause problems like memory loss and neurological disorders like neurodegenerative and neurodevelopmental disorders (Berman & Bayati, 2018). Aluminium is a neurotoxic substance associated with increased risk of Alzheimer's disease (Exley & Clarkson, 2020). Al may also cause cognitive impairment and neurological disorders. The risk of developing any neurological disorders is higher in individuals with kidney problems

because kidney is important for Al excretion (Gonzalez-Weller *et al*., 2010).

2.3.4.1. Sources

Aluminium is available in environment due to its natural and anthropogenic processes. The amount of aluminium in food is affected by a range of factors arising from various sources. While natural levels of aluminium are in foods, differences in concentration exist due to food additives and aluminium-based cooking utensils (Gourier-Frery & Frery, 2004). Aluminium is available in drinking water, and the amount of aluminium in vegetables and fruits is determined by irrigation water, soil type, and plant variety. Al in soil is transferred to plant sections during its growth. Some nuts have increased Al concentration in roots, while walnuts accumulates more Al in the leaves (Schmitt *et al.*, 2016; Singh *et al.*, 2017). Furthermore, contamination caused by human activities is also a source of Al in the marine environment (Salvo *et al.*, 2016). Al accumulates in fishes and other organisms; however, this is dependents on so many factors including species, sex and age (Salvo *et al.*, 2016).

2.3.4.2. Metabolism

Absorption: Aluminium (Al) is absorbed at the rate of 0.1 to 0.3 percent in upper intestine (Sjogren *et al.*, 2007); the absorption is higher in this part due to lower Ph (Femandez-Maestre, 2014). The amount of aluminium absorbed from foods and beverages is dependent on a numbers of factors. Several studies reported that, when citrate and fluoride are available, aluminium absorption is increased (Sjogren *et al*., 2007; Femandez-Maestre, 2014). Silicon and calcium salts are frequently used as additives in food; Due to the formation of insoluble materials with aluminium in the presence of silicon and calcium, aluminium absorption is reduced (Femandez-Maestre, 2014).

Transport and distribution: Al binds to transferrin molecules after absorption and passes through the blood-brain barrier. Since Al^{3+} has the same oxidative state as serum iron (Fe^{3+}), it can bind to the transferrin molecule (Harrison & Arosio, 1996). Transferrin-bounded Al molecule enters the cell and binds to transferrin receptors. This results in Al-transferrin complex found within the cytosol, where it binds to transferrin receptors and experiences a pH reduction to 5.5, after which Al^{3+} is released from the complex (Crichton *et al.*, 2002). Aluminium interacts with some essential elements like calcium and iron. Aluminium can replace Ca, causing demineralization and impair bone

cell growth (Mailuche, 2002; Davidson *et al*., 2007). In addition, study reported that Al may improve the stability of IRP2 (Iron Regulatory Protein-2), which regulates iron levels (Davidson *et al*., 2007). Al toxicity may thus affect bone mineralization and erythropoiesis.

Excretion: Al is excreted from the body through various routes including faeces, urine, sweat, skin, nails and semen (Genuis *et al*., 2011). However, faeces serve as the major route for excretion of non-systemic but urine is the primary route of systemic Al excretion (Genuis et al., 2011). Aluminium excretion in feces is reported to be about 74 to 96 percent of the ingested volume, according to a previous report (Krewski *et al*., 2007). It may then be inferred that GIT and renal dysfunctions may precipitate Al toxicity, especially in the vulnerable group.

2.3.4.3. Neurotoxicity

Aluminium (Al^{3+}) has high affinity for proteins, which causes Al to cross-link (Weisser et al., 2015). Unlike other elements like zinc, selenium and calcium, aluminium does not perform any known physiological function in the human body. However, the pro-oxidant and pro-apoptotic ability of Al could be responsible for its contribution to several neurodegenerative and neurodevelopmental disorders (Fig. 2.18) (Inan-Eroglu & Ayaz, 2018). In the past, the commonest reference for clinical neurotoxic effects of Al was in dialysis patients who were administered Al salts as phosphate binder dialysate; the resulting neurotoxicity observed in these dialyzed subjects was then linked to the Al (Weisser et al., 2015).

2.3.4.4. Aluminium and Autism Spectrum Disorders

Human exposure to aluminium has been associated to ASD in various research (Mold *et al*., 2018). Hair was a common specimen used to measure human exposure to aluminium in many studies, while blood and urine have only been used to a small degree (Bencko, 1995). Animal models were not left out in studies to investigate the link of ASD with aluminium (Sheth *et al*., 2017). In a recently conducted human brain research, it was reported that intracellular aluminium is associated with non-neuronal cells in autistic brain tissue. This suggests that origin and concentration of Al play a very critical role in the pathogenesis of ASD (Mold *et al*., 2018).

2.3.5. Manganese (Mn)

Manganese (Mn) is an essential component of human health. Mn is available in a wide

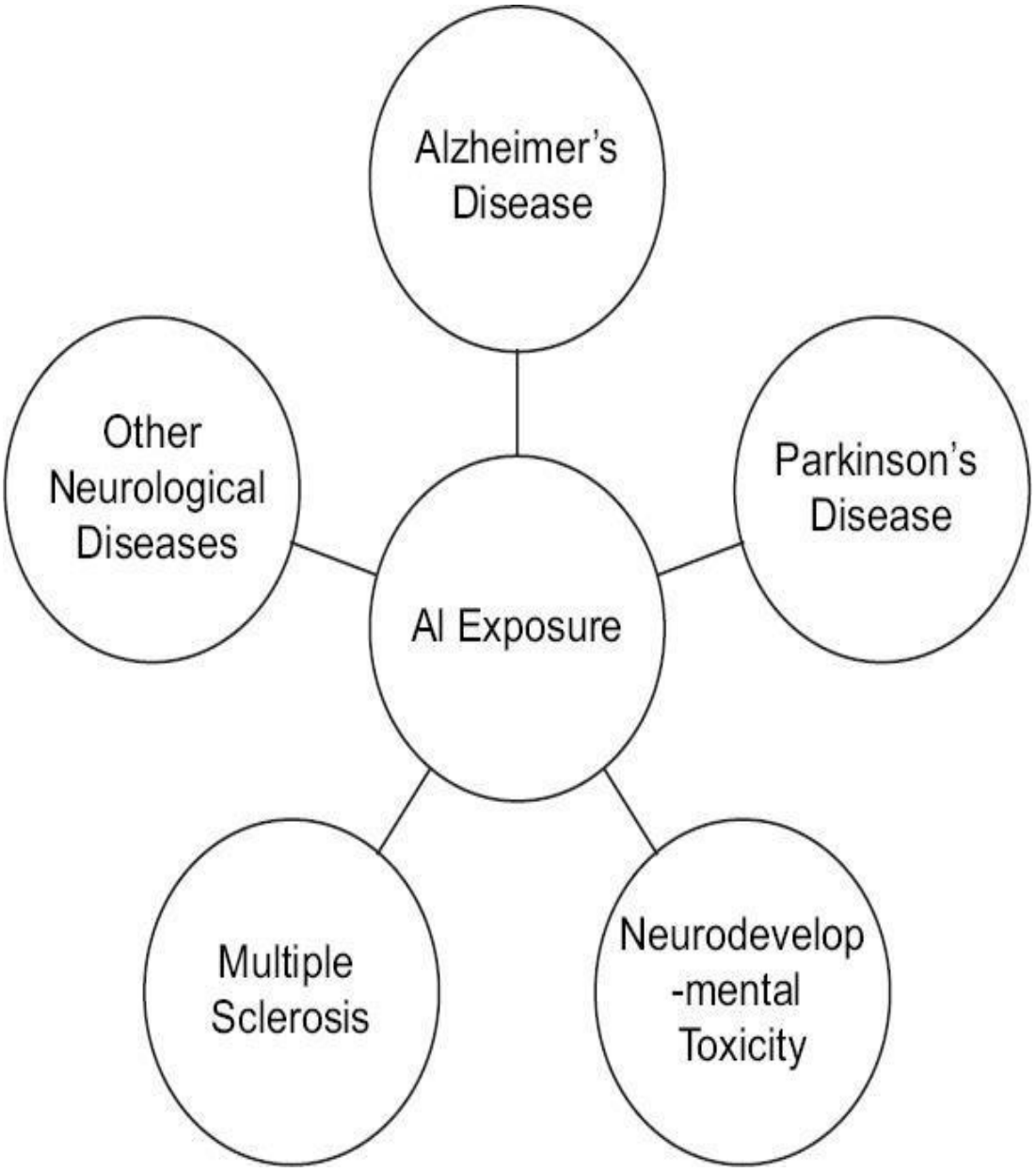


Figure 2.18: Summary of neurotoxicity of aluminum (Inan-Eroglu, E., & Ayaz, A., 2018)

range of compounds, including carbonates, silicates and oxides. Mn is naturally distributed in the rivers through erosion, and eventually finds its way into the food chain. Mn is involved in a number of physiological functional activities like reproduction, immunity, energy metabolism, growth, development as well as antioxidant defenses (Akatsu et al., 2012). Mn is needed for immune function, blood glucose metabolism and defense mechanism for reactive oxygen species (ROS) in the human diet. Mn beneficial effects are mainly based on Mn addition in metallo-proteins, which acts as isomerase, hydrolases and transferases (Horning et al., 2015; Aschner & Erikson, 2017). Mn is also known to be involved in the brain generation of glutamine from glutamate, which is carried out by glutamine synthetase (Szpetner *et al*., 2016). Glutamate is one of the neurotransmitters, and its dysfunction has been postulated to result in some neurodevelopmental disorders; hence, assessment of Mn levels may be of significance in the understanding of some NDDs.

2.3.5.1. Dietary Sources

Mn levels are highest in some readily available foods like legumes, nuts, whole grain as well as rice. Other food sources that have Mn are green leafy vegetables, seafood, spices and fruits (Horning *et al.,* 2015). Mn levels are easily obtained as a result of its presence in various sources (Horning *et al.,* 2014). Mn consumption in the average diet is around 0.9-10 mg Mn/kg/day. The highest amounts of Manganese are found in whole grains, rice, and nuts (Horning *et al.,* 2014). For children, the most essential source of Mn is breast milk, which has a concentration ranging from 3 to 10µg Mn/l. Commercial infant formulas have much higher Mn levels. Soya base milk contain higher Mn than cow's milk-based formulas (Frisbie *et al.,* 2019).

2.3.5.2. Metabolism

Absorption: About 3–5 percent of Mn consumed is absorbed in adult human. Adult males absorb 1.35 ± 0.51 percent of consumed meal with about 1mg Mn; this value is less compared to adult females who absorbed 3.55 ± 2.11 percent of consumed meal containing 1mg Mn, according to radiolabeled 54 Mn uptake studies. Mn, after digestion, is readily absorbed in intestine, although the molecular mechanisms underlining Mn absorption are unknown (Horning *et al.,* 2014).

Transport and distribution: Although Mn enters the bloodstream after leaving the GIT, the mechanism of this transport is unknown (Bhang et al., 2013). However, the

rapid distribution of plasma Mn to tissues is well-established (Bhang *et al*., 2013). Half-life of Mn in plasma is calculated to be 1 minute. Soft tissues have the largest percentage (58.11) of plasma Mn; about 30% plasma Mn are transported to the liver, 5% to both kidneys and pancreas each, 1% to the colon, 0.5% to the bone, 0.2% each to the urinary system and erythrocytes, and 0.1% to the brain (Bhang *et al*., 2013).

Excretion: The ingested Mn is rapidly depleted, within 10 days after ingestion. Excess Mn is excreted through the intestine after bile conjugation in the liver. In the entero-hepatic circulation, bile-Mn conjugates are reabsorbed in small quantities. Mn is found in trace level in urine, sweat, and breast milk (Bagga & Patel, 2012).

2.3.5.3. Deficiency

Deficiency of Mn is extremely rare, and it has not been documented in any non-experimental literature work, probably due to the abundance of dietary sources (Bhang *et al.,* 2013). Impaired development, improper bone structure and skeletal deformations, glucose intolerance, lipids and carbohydrates metabolism disruptions are all symptoms of insufficient Mn intake in the diet (Finley J.W., 2009) (Fig. 2.19). Also, elevated calcium, phosphate and alkaline phosphatase concentration were reported in individuals on Mn-deficient diet. This is suggestive of bone remodeling (Bhang *et al.,* 2013).

2.3.5.4. Manganese and the Nervous System

Mn has the capability of crossing the blood-brain barrier (BBB), though the mechanisms are still unknown. Several carrier proteins, whose identities are unknown, may be actively or passively involved in Mn transfer across the BBB. Transferrin may be a significant pathway for Mn ion influx through the BBB. Mn-citrate passage through the BBB may be based on carrier-mediated transport. Also, Mn influx in the brain may be associated store-operated calcium channels (Ayton *et al.,* 2013).

Many parts of human brain including caudate nucleus have Mn in highest concentration, while brain part like the medulla has the lowest level, according to research (ATSDR, 2012. In Parkinson's disease- brain, significantly higher Mn levels were found, especially in the putamen, while lower Mn levels were reported in the globus pallidus (Akatsu *et al.,* 2012). Mn^{3+} exposures are reported to cause higher Mn levels in the brain than Mn^{2+} exposures; thus, the type of Mn and its oxidation state may have an effect on its absorption and distribution in the nervous system (Horning *et al.,* 2015). The most abundant mangano-protein, glutamine synthetase (GS), is found primarily in astrocytes,

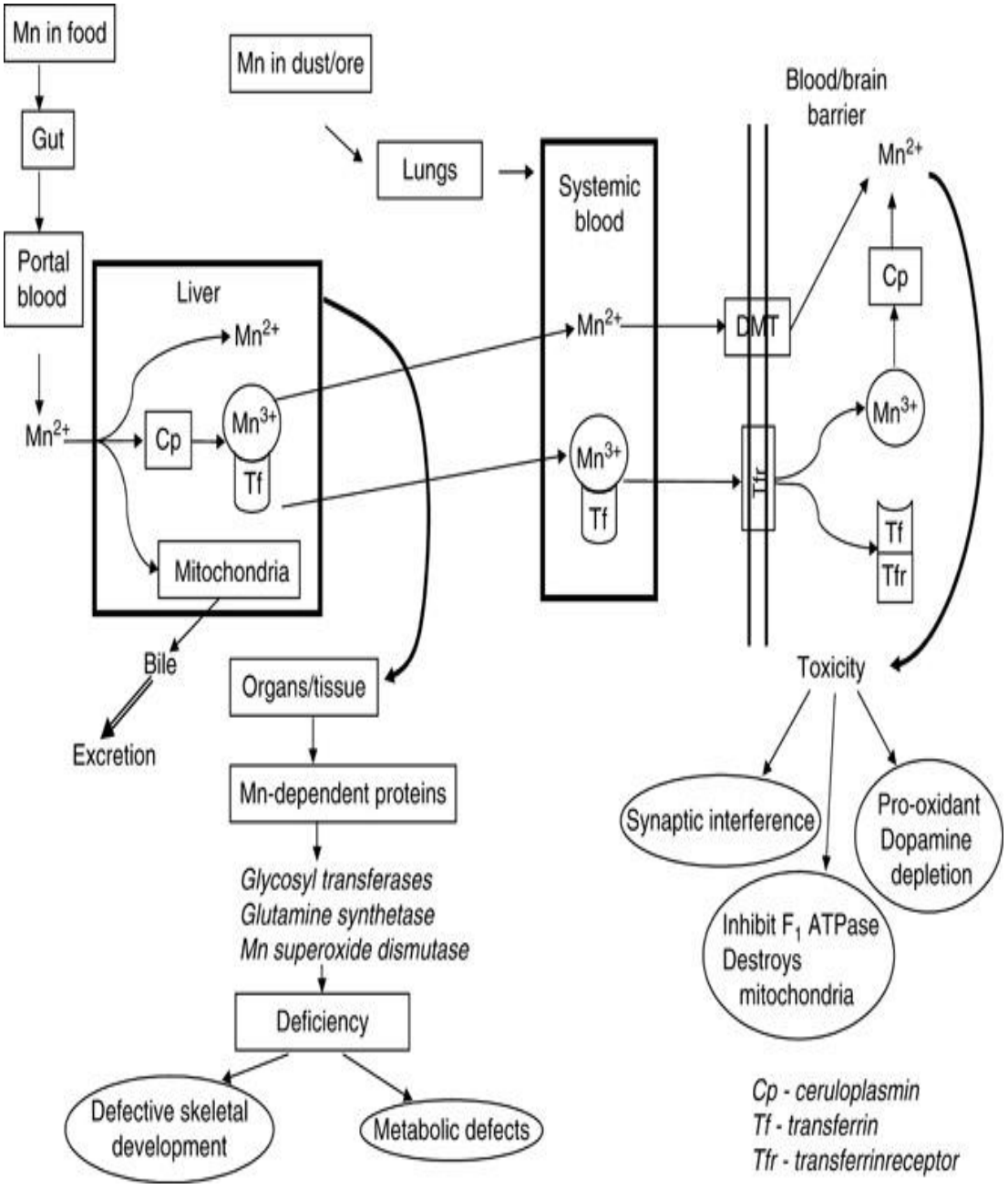


Figure 2.19: Manganese Deficiency (Finley J.W., 2009)

where it transforms glutamate to glutamine ((Eid et al., 2013). Since each octamer of GS produces four Mn ions (Eid et al., 2013). GS activity within the body is said to be regulated by Mn. Mn deficiency is reported to increase in glutamate trafficking, excitotoxicity and glutamatergic signaling (Lee et al., 2017). Also, Mn deficiency is suggested to increase seizures vulnerability and be attributed to lower level of GS (Das et al., 2019).

2.3.5.5. Manganese and Autism Spectrum Disorders

Numerous researchers examined the possible associations of ASD with manganese exposures employing the use of hair as specimen (Frye *et al*., 2020). The reports are contradicting. Some reported lower levels of Mn in enamel than in primary tooth of ASD children compared controls (Adams *et al*., 2013; Rahbar et al., 2014). De-Palma and his colleagues found no differences in hair manganese distributions between ASD and controls (De Palma *et al*., 2012). Some of these conflicting reports on ASD and other neurotoxic disorders have made diagnosis and clinical management of the disorders difficult, even in developed countries.

2.3.6 Zinc (Zn)

Zinc (Zn) is available in tissues of the body, and is involved in a variety of physiological and biochemical processes. In 1961, Zn deficiency was discovered as a personal health challenge; this spurred researchers' interest to investigate the biochemical and clinical relevance of zinc (Hambidge *et al.,* 2011). Although many studies were carried out on biochemical mechanisms of zinc-dependent physiologic functions, no clear relationship has yet been fully established based on its ubiquitous nature. Zinc is ubiquitous within cells, and its role is categorized into catalytic, structural and regulatory based on functional groups (Roohani *et al.,* 2013). Zinc is needed for metabolism of carbohydrate, lipid, protein and nucleic acid and other essential elements (Intorre *et al.,* 2008). It is found in over 300 enzymes (Cheng & Chen, 2021). Cells preservation and organs integrity maintenance requires Zn. Again, Zn plays a very important function in polynucleotide transcription and is thus involved in gene expression processes. Zinc's role in the immune system is central and important, it has a great effect on humoral and cellular immunity (Bonaventura *et al.,* 2015).

2.3.6.1. Dietary Sources

Zinc concentrations are highest in common foods like lean meat, red meat, legumes and

whole-grain cereals. Zinc content ranges from 10 to 25 mg/kg (150 to 380 mmol/kg) in processed cereals or high-fat meat (Sharma *et al.,* 2013). Zinc is available in small concentration in foods like fish, green leafy vegetables, and fruits [10 mg/kg (150 mmol/kg)]. Also, zinc content is very low in separated fats and oils, sugar, and alcohol (Murphy *et al.,* 1975; Sharma *et al.,* 2013).

2.3.6.2. Metabolism

The summary of zinc metabolism is shown in Fig. 2.20

Absorption: The small intestine is where zinc is absorbed most efficiently, and the absorption of Zn is proportional to its concentrations (Hunt & Beiseigel, 2009). The absorption in the small intestine takes place via a mechanism called carrier-mediated transport. Zinc absorption increases as dietary zinc levels rise to maximum. Individuals that are deficient in zinc absorb it more efficiently, whilethose who consume a high-zinc diet absorb it less efficiently (Hunt & Beiseigel, 2009).

Transport: There are minimum of ten zinc transporters (ZnTs) and fifteen Zrt-/Irt-like proteins (ZIP) transporters in human cells. In cellular zinc homeostasis, they play opposing roles. ZIP transporters increase availability of the intracellular zinc by increasing extracellular zinc absorption via increase release of vesicular zinc into the cytoplasm; on the other hand, ZnTs reduce availability of the intracellular zinc through the enhancement of zinc efflux from cells into intracellular vesicles (Kambe et al., 2015). Cellular Zn absorption is controlled by an intracellular metal binding protein-metallothionein, which works in tandem with the ZnTs (Kambe *et al*., 2015). Various factors, such as dietary zinc supplementation and intraperitoneal zinc promote MT synthesis in the liver and intestine (Kambe *et al*., 2015).

Homeostasis: Zinc homeostatic balance is primarily maintained through changes in direct absorption and intestinal excretion of Zn in both human and animals (King, 2000; King, 2011). Endogenous intestinal excretion appears to respond in time to intake changes that are above or below optimal, while zinc absorption appears to take more time to respond. It does, however, have the potential to accommodate significant variation in intake (Veenemans *et al.,* 2011).

Excretion: Excretion of Zn in the body is mainly through the gastrointestinal tract. Nearly 50% of the body Zn is lost via the GIT. Sweat and urine are two other ways that zinc is excreted (Roohani *et al.,* 2013). As a result, an abnormality in the GIT may affect Zn

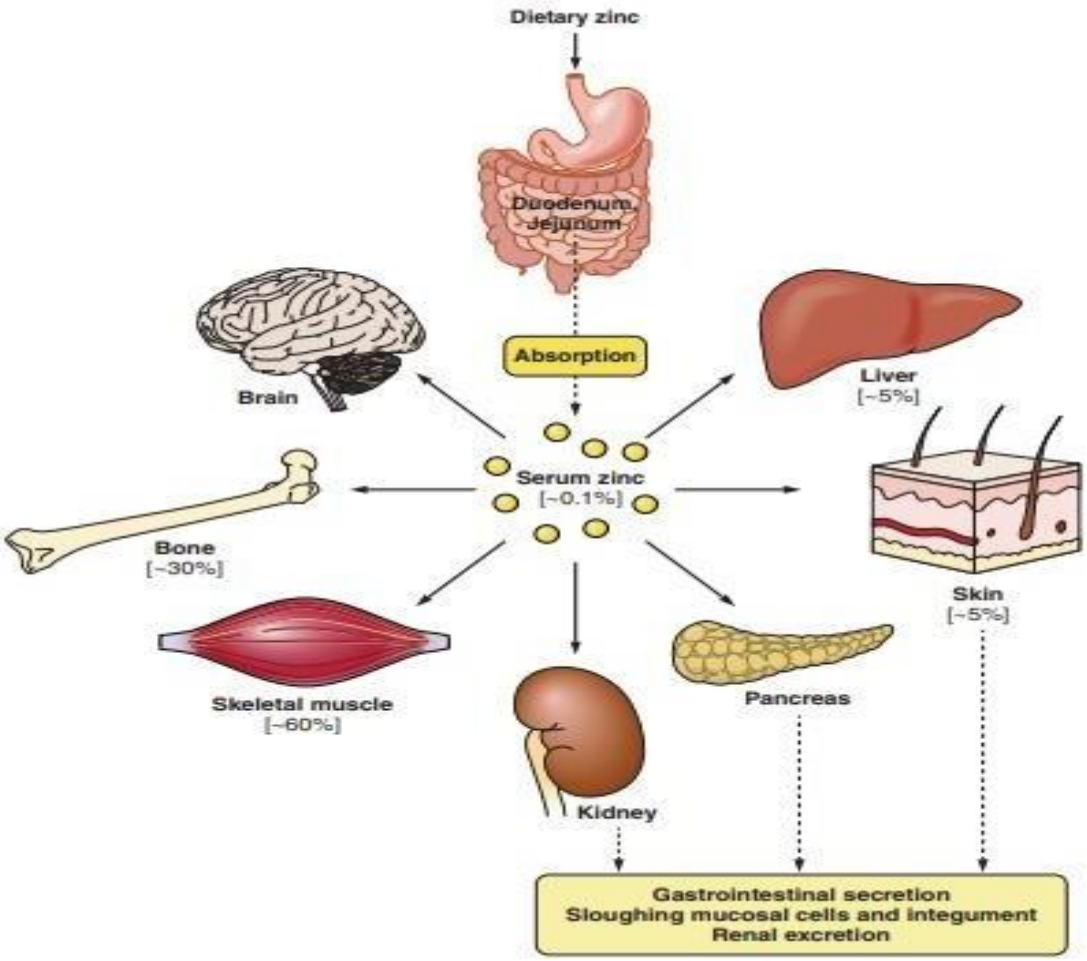


Figure 2.20: Metabolism of Zinc (Kambe, T., Tsuji, T., Hashimoto, A., & Itsumura, N. (2015).

levels in the body.

2.3.6.3. Zinc Deficiency

Zn deficiency is known to clinically affect some important systems of the body; these include reproductive, immune, central nervous systems as well as skeletal and gastrointestinal systems (Schlemmer *et al.,* 2009). Growth and development are also impeded by zinc deficiency. When zinc needs are highest, such as during pregnancy, infancy, and puberty, these effects are most apparent (Roohani *et al.,* 2013). Zinc deficiency in children is associated with risk of serious infectious diseases, based on findings from zinc supplementation studies (Black, 1998; Black, 2003; Dhingra *et al.*, 2009). In zinc-supplemented classes, acute diarrhoea episodes were shorter in period and magnitude and the frequency was reduced. In otherstudies, zinc supplementation was reported to minimize acute lower respiratory tract infections and malaria incidences (Nair *et al.,* 2017; WHO, 2011). Preventing zinc deficiency and suboptimal zinc status in children by increasing zinc intake and availability can solve major impact Zn deficiency has on the health of children in the developing countries (Black, 1998).

2.3.6.4. Causes of Deficiency

Inadequate zinc consumption, increased body needs, malabsorption, increased losses, and impaired body use are all causes of zinc deficiency (Veneman *et al.,* 2011). In most cases, Zn deficit is attributed to a low to none absorbable zinc in food. This may be due to a lack of zinc infood or a strong dependence on foods with little to low zinc absorption (Veneman *et al.,* 2011). Zinc deficiencyin the diet is very common, and physiological conditions linked to elevated zinc requirements often aggravate it (Veneman *et al.,* 2011).

Secondary zinc insufficiency may occur due to malabsorption syndromes and inflammatory bowel diseases, which cause poor zinc absorption and loss. This is the primary deficiency. Zinc use is impaired in the presence of infection due to reduced zinc distribution, which decreases zinc supply to tissues. Zinc absorption is decreased and endogenous zinc losses are increased when intestinal integrity is impaired (Veneman et al., 2011). Also, zinc excretion in the stool is increased during acute diarrhoea (Hunt & Beiseigel, 2009).

2.3.6.5. Zinc and Autism Spectrum Disorders

Neuropsychological changes like emotional distress and irritability may occur in people

with severe Zn deficiency (Hambidge, 2000). The role of Zn in neurodevelopment is quite significant. Zn has been associated with both neurotransmission and cognition. Zinc deficiency affects cognitive development and performance and is also involved in long- and short-term glutamatergic transmission effects (Lang *et al*., 2015). Zn uptake is needed for glutamatergic neurotransmitter activation, and synaptic neurotransmission (Blakemore & Trombley, 2017). Zn tends to act as a co-transmitter for glutamate, since it was formerly deposited into the secretory granules, which then released glutamate into the synapse (Blakemore & Trombley, 2017). The incidence of zinc deficiency and copper toxicity is high in ASD children (Sayehmiri *et al.,* 2015). When comparing low functioning autism group children's hair and nails to a control group, Lakshmi Priya & Geetha (2011) reported a substantial difference in Zn level in hair and nails. Although the actual biochemical mechanism of Zn in neurodevelopment has not been elucidated, several studies have associated Zn deficiency with autism. One of the hypotheses of these studies was that Zn deficiency in ASD is a secondary consequence of elemental imbalance, resulting in metabolic disturbances and oxidative stress (Bjorklund, 2013).

2.3.7. Selenium (Se)

Selenium (Se) is one of the important trace minerals and elements needed for human health, but it is only needed in trace quantities (Thomson, 2004). Selenium is integrated into about 25 enzymes, including glutathione peroxidases, iodothyrodine deiodinases, thioredoxin reductases, and selenoprotein-P, to form selenoproteins, which are essential antioxidant enzymes (Zoidis et al., 2018). Selenoproteins' is an antioxidant which helps in protecting cells of the body from free radical damage (Zoidis *et al*., 2018). Other selenoproteins are involved in thyroid activity and the immune system (ANA, 2010).

2.3.7.1. Sources

The main dietary sources of selenium are plant foods. Plants derive their Se from soil on which they are grown. Hence, the selenium content in any food like vegetables is affected by the type of soil which the plants are grown (Longnecker *et al.,* 1991; ANA, 2010; Gupta & Gupta, 2017). Even when grown in seleniferous soil, vegetables including peas, tomatoes and cucumbers contain a maximum of 6mg per gram/selenium (Mehdi *et al.,* 2013).Onions and asparagus, for examples, contain about 17µg/g of selenium when planted on such soils (Mehdi *et al*., 2013). Garlic and brassicas and others in that group serve as very good sources of selenium. Selenium levels in fruits are typically low, rarely reaching 10 µg/kg (Mehdi *et al*., 2013). Selenium is also present in

a range of meats and seafoods. It was reported that highest selenium levels are available in the muscles of animals that feed on plants grown on selenium rich soil (ANA, 2010). Selenium content of food from animal sources is determined by the animals' diets and soils in which they are raised. Dietary selenium is commonly found in meats and bread in the United States (Terry and Diamond, 2012). Some nuts are also good sources of selenium (ANA, 2010). Milk has a relatively low selenium content (about 0.05 ppm). Dietary supplementation, on the other hand, will increase the selenium levels. Selenomethionine is the most common source of seleniumin human feed. Selenocysteine and selenite concentrations in human diet are relatively low; however, sodium selenite, potassium selenate, and barium selenite are the common types of oral selenium found in supplements (Mehdi *et al.,* 2013).

2.3.7.2. Biological Functions

Many animals, including humans, need selenium as a trace element. Selenium is important component in glutathione peroxidase enzymes as well as a number of other proteins. There are 25 selenoproteins in the human selenoproteome (Croft *et al.,* 2007). These include SeCys, about half of which have biological roles (Zoidis *et al.,* 2018). Selenoproteins in bacteria are for catabolic processes, while selenoproteins in mammals are understood to be involved in antioxidant and anabolic processes (Zoidis *et al.,* 2018). GSH-px enzymes, which are selenium-dependent, protect the body from oxidative stress by lowering lipid peroxides and hydrogen peroxide. It was recently discovered that a cardiovascular disease risk factor, homocysteine, inhibits GSHpx1 (Loscalzo & Handy, 2014). Other selenoproteins known as thioredoxin reductases catalyze the removal of oxidized cellular proteins and may make a significant contribution to the outcome of oxidative stress, redox control as well as apoptosis resistance (Zhang *et al.,* 2020).

2.3.7.3. Metabolism

Glutathione (GSH) is a crucial part of selenium metabolism (Mehdi *et al*., 2013). It is involved in a number of reduction activities. These reactions convert selenite to hydrogen selenide (H_2Se), which ensures a steady active supply selenium for selenoprotein synthesis. The late trimethylselenonium ion [$(CH_3)_3Se^+$] is formed by a series of sequential methylations of H_2Se (Mehdi *et al*., 2013). Selenium is absorbed very slowly until it reaches the intestines. The majority selenium compounds absorbed in the GIT are water-soluble selenium compounds.

Transport and distribution: The duodenum and large intestine are the primary sites of selenium absorption. It occurs primarily through active transport via a sodium pump. There are no clear pathways for selenium absorption in the intestine, but they differ depending on the element's chemical type. Organic forms, i.e., selenomethionine and selenocysteine, follow the amino acid absorption mechanism. Selenite absorption is through easy diffusion, while Selenate is absorbed through a co-transport of sodium selenate and exchange selenite/OH system. Selenomethionine is absorbed by an active mechanism in the small intestine, similar to way methionine is absorbed (Mehdi *et al.,* 2013). The rate of selenium absorption is reported to be slowed by certain elements, including sulfur, lead, arsenic, calcium, and Fe^{3+} (Bhattacharya *et al.,* 2016). Sulfur reduces selenium absorption due to steric competitiveness, while Fe^{3+} precipitates selenium into a complex form that is indigestible by enterocytes (Vendeland *et al.,* 1994; Spears *et al.,* 2008). Hence, excess of these elements may also predispose humans to Se deficiency.

Excretion: In most instances, urine is the primary excretory route for Se, but the percentage excreted in the urine is dependent on ingested dietary selenium concentration, food compositions, the chemical form, the selenium types in the individual, and the percentage amount of the glomerular filtrate (Pedrosa, *et al*., 2012). Excretion through exhaled breath becomes essential, if not dominant, at high or toxic dose concentration (Alexander, 2015). The data on selenite biotransformation and excretion supports the theory that methylated selenides formed from biotransformation process is primarily used in promoting toxic amounts of selenium excretion (Pedrosa, *et al*., 2012).

2.3.7.4. The Nervous System and Selenium

Selenium (Se) is known to play essential role in brain health (Gashu & Stoecker, 2017). Many studies have shown that the element has an impact on different central nervous system pathologies (Moghadaszadeh & Beggs, 2006; Ye *et al.,* 2021). Low levels of plasma Se are linked to decrease in neurological function like coordination in elderly patients (Shahar *et al.,* 2010). Se has been shown to affect behavioural development as well as psychological factors like mood and perception (Watanabe & Satoh, 1994; Steinbrenner & Sies, 2013). Se is an organic compound which can have antipsychotic properties (Machado *et al.,* 2006). Equally, Se has neuroprotective effects (Watanabe & Satoh, 1994; Lu *et al.,* 2014, Nazirolu *et al.,* 2014) on brain function and against various

central nervous system diseases. Research has shown that Se acts as a neuroprotective agent, which helps to prevent the development and progression of neurodegenerative disorders. However, due to complex nature of these disorders and numerous roles of Se, further research into the mechanisms by which this element interacts in these diseases is needed. More research is needed to better explain selenoprotein functions and possible implications on neurodegenerative disorders (Zhang *et al*., 2020).

2.3.8. Vanadium (V)

The element vanadium is present in the earth's crust at a level around 100 mg/kg (ATSDR, 2012). Vanadium available naturally in water, soil and air in concentrations that are not harmful to health. Vanadium is naturally found in low amounts in most foods, with sea foods having higher concentrations than meat from land animals (ATSDR, 2012). However, increased vanadium emissions to the environment are primarily linked to industrial sources. Humans are thought to release a greater volume of vanadium into the atmosphere than natural sources (ATSDR, 2012). It is not clear if vanadium is an essential trace element, and no vanadium deficiency related disease has been identified. Human intake of vanadium may be either through food, drinking water or inhalation (ATSDR, 2015). Vanadium is used as catalyst in some materials production and also in the pesticides manufacturing. Human beings are exposed to vanadium via environmental exposure from crude oil spillage and automobile exhaust (Olopade et al., 2011; Fatola et al., 2019).

It has been demonstrated that vanadium exposure, regardless of route and duration, affects glia cells as well as neurons. Folarin *et al.* (2017) observed a widespread accumulation of vanadium throughout the brain with a preference for the olfactory bulb, brain stem, and cerebellum in a study of chronic intraperitoneal vanadium exposure at 3 mg/kg in mice. The increase of lipid peroxidation in the brain after vanadium injection is evidence that oxidative stress contributes to the metal's neurotoxicity (Folarin *et al.*, 2018; Fatola *et al.,* 2019). Olopade and Connor, (2011) have suggested that vanadium's neurotoxicity is likely caused by the induction of reactive oxygen species. It has also been reported that vanadium and its compounds cross blood brain barrier and cause neurological consequences (Olopade and Connor, 2011).

2.3.8.1. Food Sources

Vanadium is naturally found in low amounts in most foods, with seafood containing higher levels than meat from land animals. Dietary vanadium intakes have been reported

to be around 0.01-0.02 mg/day (Cseh *et al*., 2012). Vanadium in drinking water has an average amount of 0.001 mg/litre. Vanadium is poorly absorbed (0.2 to 1.0 percent), according to human research. Fasting, dietary composition and speciation also have the ability to influence the absorption of vanadium. Vomiting was reported in participants who ingested about 7.8-10mg vanadium/24hours for 2 weeks, while increased doses of about 14-42mg vanadium/24hours for 2 weeks showed gastrointestinal symptoms like anxiety, cramping, diarrhea and vomiting (Zhu *et al*., 2016).

2.3.8.2. Metabolism

Vanadium is absorbed by two primary routes: breathing and ingestion, each of which can be hazardous depending on the dosage (Treviño *et al.*, 2019). The lungs are the primary site of vanadium toxicity in the environment (Cooper, 2007). The rate of vanadium uptake in the respiratory system is influenced primarily by the vanadium compound solubility rate and particle size. Vanadium exerts a strong effect on human bronchial tube smooth muscle after inhaling vanadium-containing compounds, triggering spasms by encouraging Ca^{2+}release from the stored Ca intracellularly through the inositol phosphate production and Ca^{2+}-ATPase inhibition (Rehder, 2013). This may have an indirect effect on Ca^{2+} homeostasis.

Ingestion is another important route for vanadium absorption. Less than 5% ingested vanadium is absorbed by the intestinal tract based on daily vanadium intake, urine and feces amounts. Oral absorption of vanadium is mostly caused by two types of vanadium: vanadates found in drinking water and vandal (Gad & Pham, 2014).

Since Ca^{2+} ATPase is a crucial enzyme required in the regulation of entrance of nutrients through the cell membrane, inhibition of this enzyme may affect this process. Such effect may be deleterious to neurodevelopment, especially in a developing fetus (Sanders *et al.*, 2009). The physiological role of vanadium and its contribution to redox stability and oxidative stress is depicted in Fig. 2.21.

Transport and distribution: During the administration of vanadium compounds, vanadium is exposed to a variety of factors before entering the bloodstream. As a result, prior to being bio transformed into biologically active forms that circulate in blood plasma, they are solubilized on getting to the alveoli or when exposed to an acidic environment within the intestine. Once vanadium enters the bloodstream, it binds to plasma protein, especially albumin and transferrin. Vanadyl also binds negatively

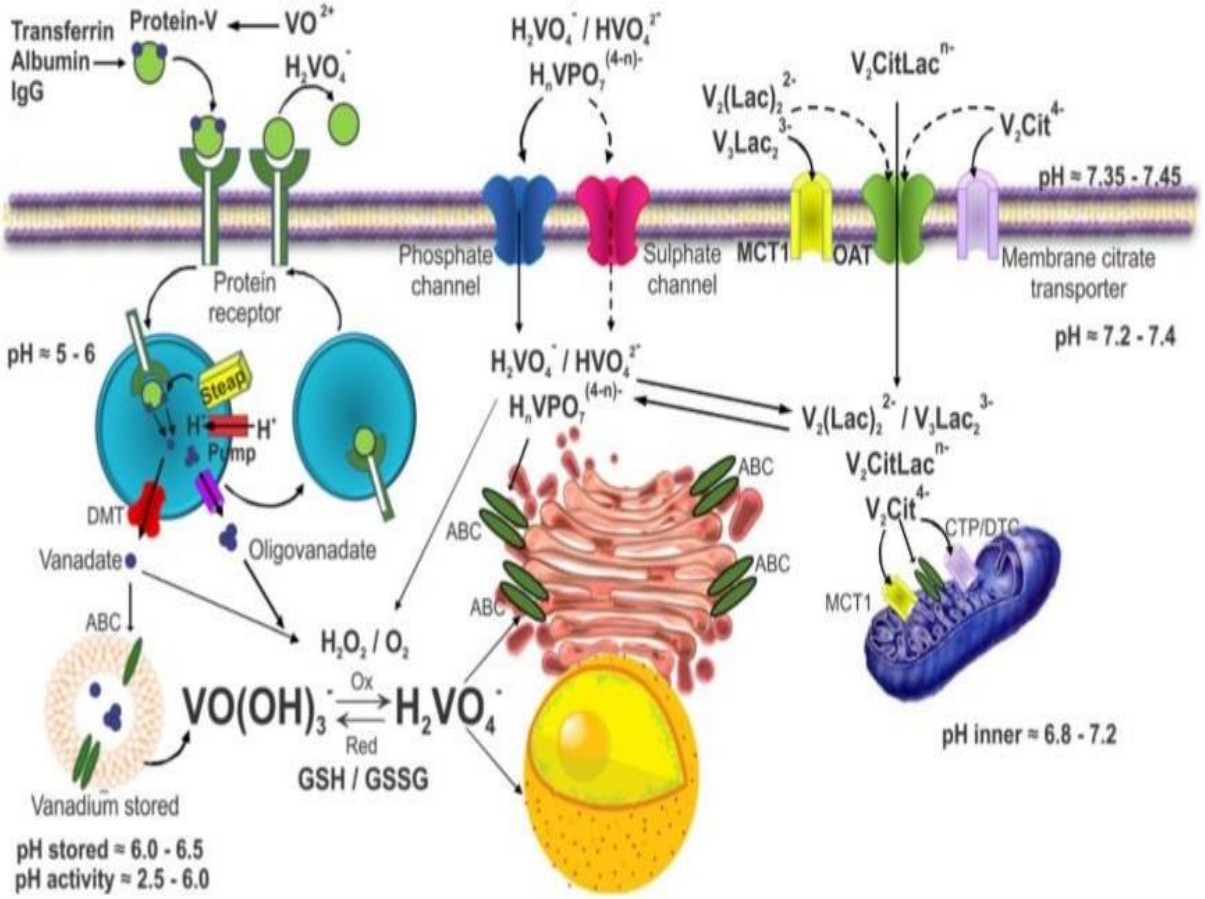


Figure 2.21: Redox equilibrium, oxidative stress, and vanadium interconversion species (Render, 2012).

charged serum molecules like glycine, histidine, oxalate, lactate and phosphate just as it binds proteins (Treviño *et al.,* 2019).

In the bloodstream, vanadium attach to transferrin at biologically optimal concentration, where the vanadyl ion replaces Fe^{3+} ion binding sites (Correia *et al.,* 2017). The displacement of Fe^{3+} may be a competitive inhibition process for the latter and possible negative physiological process. Transferrin has affinity for vanadium carrier to albumin because it has a metal binding site (Liboiron *et al.,* 2005). In the transferrin complex, vanadium has the capacity to displace 30–70 percent of iron ion stored initially in the body (Kiss *et al.,* 2006). Bloodstream vanadyl has been shown to bind to immunoglobulin G at high concentrations (Sanna *et al.,* 2012; Sanna et al., 2017). Furthermore, certain vanadyl compounds containing insulin-enhancing effects stayed in the bloodstream for a longer time, allowing for a link between vanadium blood concentration and albumin attachment (Makinen & Salehitazangi, 2014). The oxygen-carrier protein, hemoglobin (Hb), also aids vanadium bloodstream transport. Vanadium is reduced in the erythrocyte environment, which is mainly driven by glutathione. The vanadium ion is largely bound to hemoglobin within erythrocytes, according to experimental studies (ATSDR, 2012; Lopez-Rodriguez *et al.,* 2017; Trevino *et al.,*2019), although some intracellular bioligands may compete for vanadium.

Excretion: It appears that only small quantities between 0.1 – 1.0mg of vanadium are required for people's wellbeing. Hence, most ingested vanadium is excreted in urine and faeces (Orris et al., 1983).

2.3.9. Copper (Cu)

Copper (CU) is a reddish element which may be seen in rock, soil, water, and sediment at a low concentration; it can also be found in the air and in all animals and plants (Oorts, 2013). Copper is a trace element and an important mineral for all living cells. It is important for embryonic growth, metabolism and growth in man (Tapiero *et al.,* 2003). Anemia, cognitive and neurological defects, and heart failure are all symptoms of copper deficiency. It is found naturally in certain foods and can also be obtained as a dietary supplement. Copper is used in redox reactions and can be quickly be changed to Cu^{2+} from Cu^{+} and vice versa. Many enzymes responsible for energy production, neurotransmitter synthesis and activation require copper as a cofactor (Institute of Medicine, 2001; Prohaska, 2012; Collin, 2014). Ceruloplasmin (CP), one of the most

common cuproenzymes, plays an essential part in iron metabolism, and in normal healthy human plasma, copper accounts for more than 95% of total copper (Hellman & Gitlin, 2002). Copper is thought to be essential in maintaining neuro-hormone homeostasis, brain growth, gene expression control and immune system function (Collins, 2014). The functions of copper are summarized in Fig. 2.22. Furthermore, the copper-containing superoxide dismutases are essential for copper protection against oxidative damage (NIH, 2019).

2.3.9.1. Environmental Levels and Exposure Sources

Copper consumption through food in adult ranges from 1.0 to 2.5 mg per day, equivalent to 15–45 g/kg adult body weight (EFSA, 2015). The Kidney and livers remain the richest sources of copper, both contain elevated levels of copper more than any other sources, although, it is also abundant in fish, green vegetables, fruits, nuts and cereals, while meatand dairy products like milk have the lowest levels (WHO, 2004).

2.3.9.2. Metabolism

Copper is an essential mineral but potentially harmful when present in excess amount. It functions as an electron acceptor after being converted from Cu^{+} to Cu^{2+}. It, however, becomes a toxic element when consumed in excess and thus becomes converted from Cu^{2+} to Cu^{+} (Solioz & Vulpe, 1996). The need, therefore, to control its transport and concentration in the body becomes imperative. Copper absorption, distribution, storage, and excretion have all been regulated by efficient homeostatic mechanisms (Fig. 2.23). Complexing molecules have long been identified in blood, but cellular mechanisms have remained largely unknown. Several cellular copper transporters were discovered in the last decade (Solioz and Vulpe, 1996), the first of which is ATP7A. Following ATP7A, several copper-specific transporters were discovered, all of which are involved in distribution of copper to different compartment and copper-requiring enzymes (Solioz & Vulpe, 1996).

Absorption: Cu absorption is affected by the source of exposure as well as the concerned Cu ionic type (*Tapero et al.,* 2003). There seem to be no quantitative research on copper and degree of its compound absorption after inhalation from human or animal studies. The mucosal membrane of the small intestine and, to a lesser degree, the stomach absorb copper from the diet (Wapnir, 1998). The amount of Cu in the diet tends to have the greatest effect on absorption in adults (Turnlund et al., 1989). The bulk of

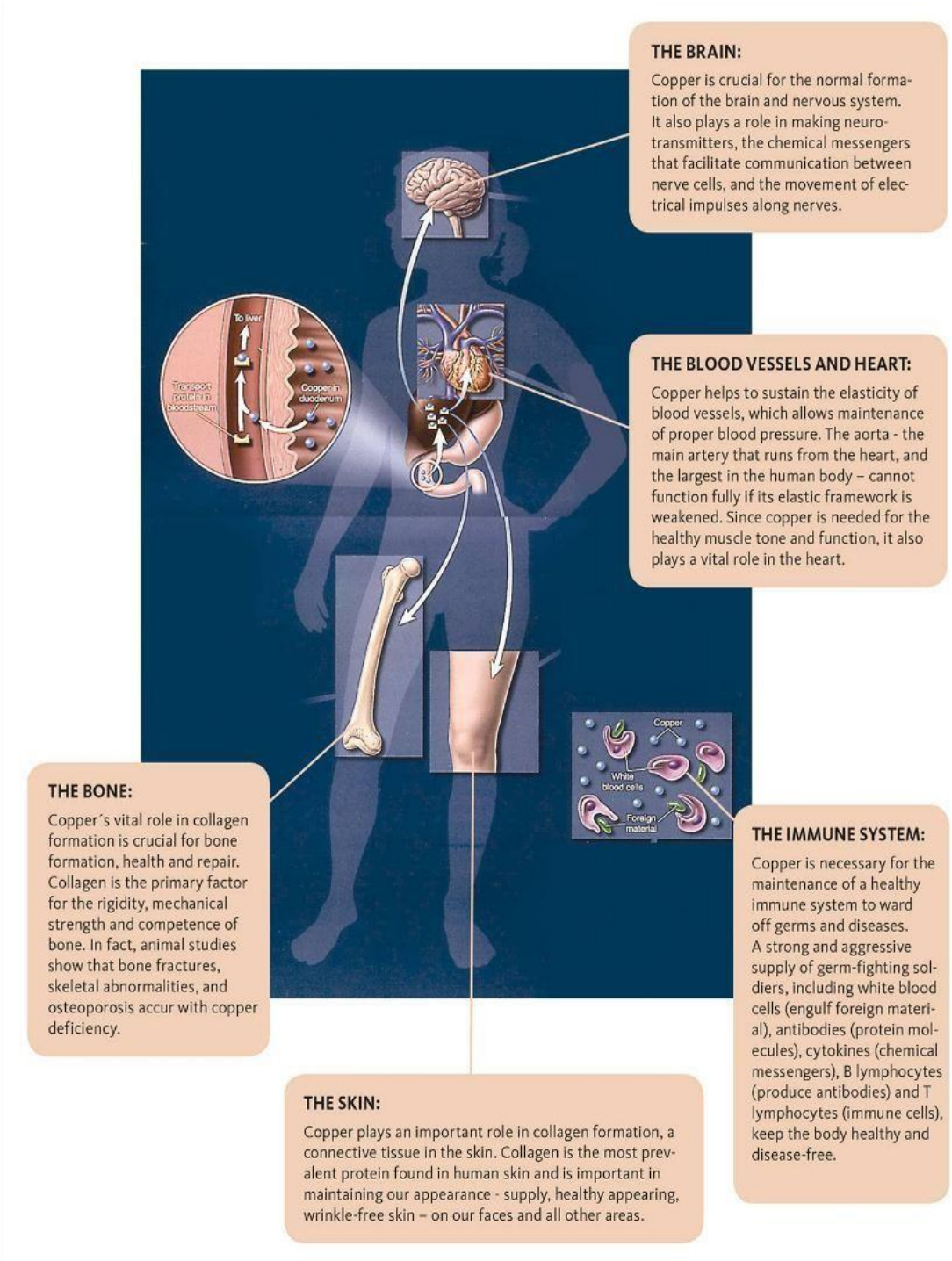


Figure 2.22: Copper: Essential for Human Health (Copperutensils, 2016)

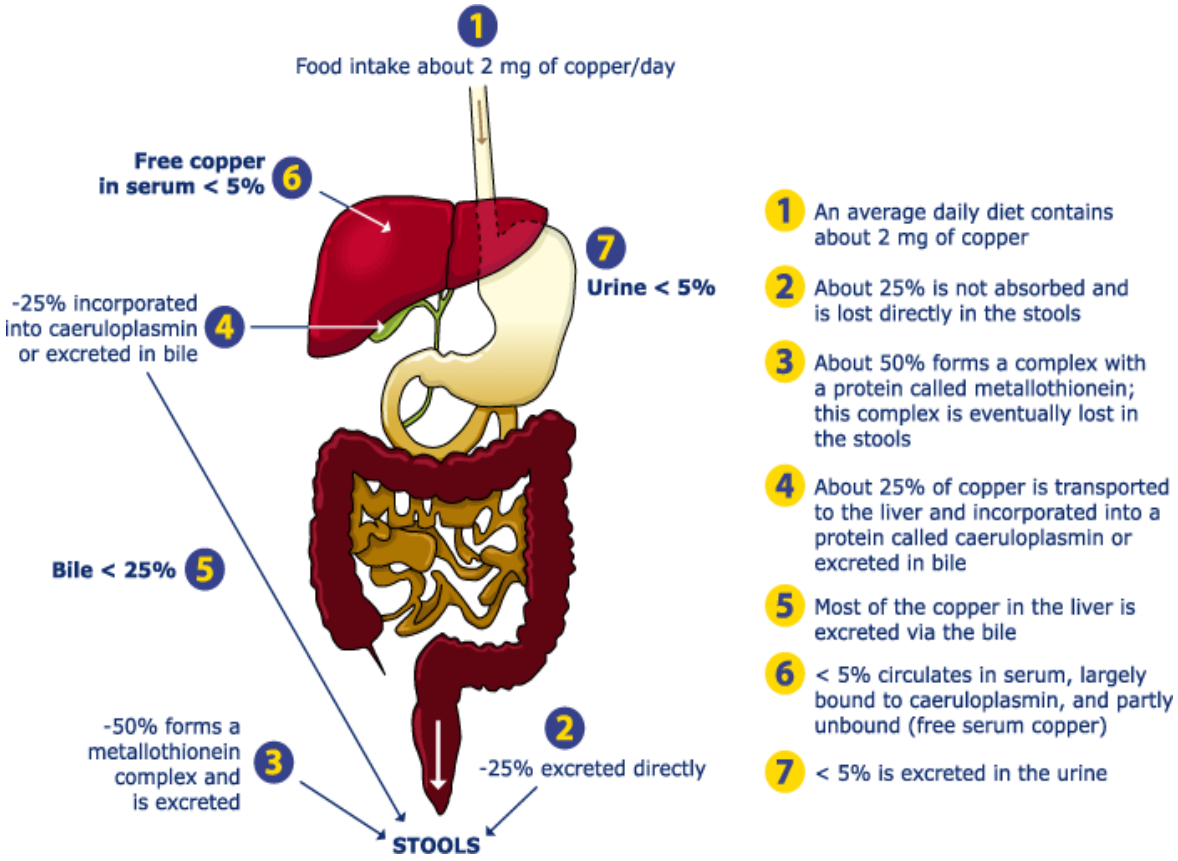


Figure 2.23: Metabolic pathway of copper (Eurowilson.org)

Cu absorbed is contained in mucosal cells as metallothionein or glutathione (Tapiero et al., 2003).

Excretion: The bile excretes about 98 percent copper, and the remaining 2 percent is excreted in urine in normal physiological situations. The liver is important in copper metabolism because it functions as a storage site and also regulate excretion of bile (Boyer, 2013). Only in severe cases, such as Wilson's disease where normal tubular reabsorption is exceeded, does renal filtration becomes essential for its excretion (Bartee & Lutsenko, 2007). Human sweat losses just a trace amount of copper (Linder & Hazegh-Azam, 1996). Also, copper is excreted in small amounts through saliva, pancreatic, gastric, and duodenal daily (Linder & Hazegh-Azam, 1996).

2.3.9.3. Copper and Nervous System

Copper is essential for the brain's as well as nervous system's normal growth. It is necessary for the development of the brain and the preservation of myelin, that protects nerve cells and ensures that nerve impulses are properly transmitted. Copper also plays a role in neurotransmitter synthesis, which are chemicals that enable nerve cells to communicate with one another. Copper deficiency can result in nervous system damage. Copper is absorbed in the brain when it is moved from the cerebral endothelium to the choroid plexus barrier in a regulated manner. Choroid plexus expresses elevated levels of CTR1, ATOX1, and ATP7A, suggesting that this tissue is involved in copper absorption and/or efflux within the cerebrospinal fluid (Zheng et al., 2014; Monnot et al., 2012). Brain copper levels are reduced when CTR1 or ATP7A are unavailable. Copper levels in Menkes babies' brains are reduced (Horn et al., 1992). ATP7B functions in brain activity, which makes it distinct from that of ATP7A. Under certain circumstances, ATP7A will take the place of ATP7B. (Barnes et al., 2005). Pineal night-specific ATPase (PINA), a type of ATP7B, is located on the retina and also on the pineal gland. This protein appears to be required for the regulation of circadian rhythms by a copper-dependent portion (Borjigin et al., 1999). The following Fig. 2.24 shows the role of ATP7B in the transportation of Cu into brain and other body organs.

2.3.9.4. Copper and Autism Spectrum Disorders

Copper may be present in many parts of the brain like basal ganglia, hippocampus, and cerebellum, among others (Madsen & Gitlin, 2007). Cu toxicity has been reported to have a significant impact on the mind based on the magnitude of severity (Priya & Geetha, 2011; Ghada *et al*., 2017). Copper is said to be involved in development of

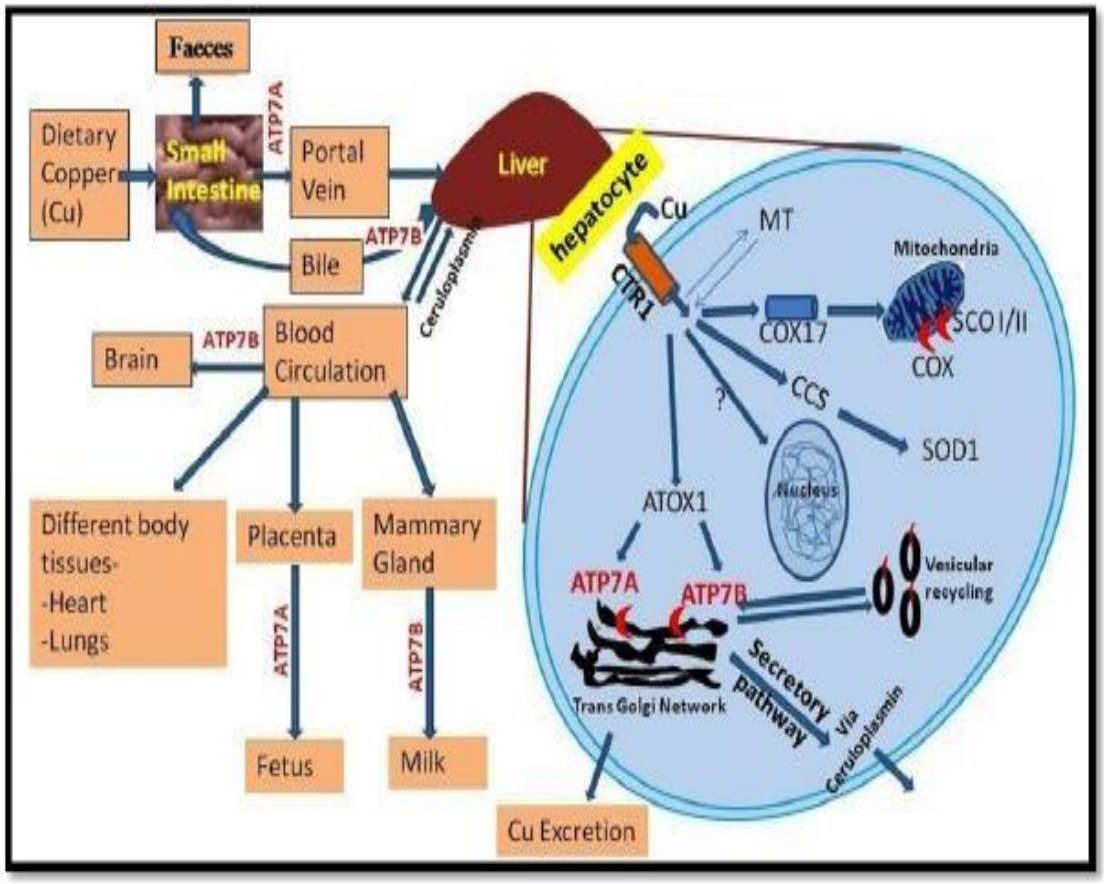


Figure 2.24: Transportation of copper in the body and hepatocytes (Prasad, R., & Kumar, S., 2013)

neurological disorders, either directly or indirectly. A disturbance in copper metabolism has been linked to ASD, and level of copper found in ASD was observed to be altered (Russo & DeVito,2011; Bjorklund, 2013; El-Baz *et al.,* 2018). Russo & deVito (2011) reported that autistic children have significantly elevated copperin comparison to control in a study. Similarly, Geetha and colleagues (2011) and SaldanhaTschinkel and research mates (2018) in their studies reported an increased copper levels in ASD compared to controls (Geetha *et al.,* 2011; Saldanha Tschinkel *et al.,* 2018). In a meta-analysis review to investigate the disorders, a study found a connection between copper metabolism and ASD. The authors stated that a significant correlation did exist between Cu levels and ASD development (Grabrucker *et al.,* 2016). The role of Cu, especially Cu (II), in neural development and the manifestation of its deficiency in Menkes disease make this element a possible contributor to the ASD pathophysiology.

2.3.10. Arsenic (As)

Arsenic is the periodic table's 33rd element and the world's 20th most common element. It is, in reality, a metalloid part of the crust of the earth that occurs naturally. It is only available in trace amounts in rocks, air, water and soil. Higher than normal concentrations could, however, result from human activities, including smelting and use of pesticides (Mandal & Suzuki, 2002).

2.3.10.1. Sources

Human comes in contact with arsenic in many ways, but industrial sources like smelting and microelectronics remains the most common ways. Wood preservatives, herbicides, pesticides, fungicides, and paints are all sources of arsenic (Sauvé & Desrosiers, 2014). Arsenic from these sources lead to contamination of water, soil and agricultural products. Following ingestion of infected food, fruits, vegetables, or drinking water, and smoking tobacco, the gastrointestinal tract becomes the most common aarsenic’s path of entrance into the body. Inhalation and dermal exposures have also been suggested in industries, including glass production, smelting, wood treatment and pesticide productionindustries (Ferreccio *et al.,* 2013). Upon entry into the body, arsenic is found in the bloodstream and accumulates in many important organs of the body, which include the heart, lungs, liver,kidney, nails, and hair (Khan *et al.,* 2022).

2.3.10.2. Toxicity

Human and animal have been shown to be affected by the inorganic forms of arsenic. These inorganic forms of arsenic tend to react with the body's cells, displacing some elements and thereby affecting cell function. Acute or chronic poisoning from arsenic exposure is possible. Acute arsenic poisoning, which occurs when a high dose of arsenic is consumed, causes the disruption of blood vessels and gastrointestinal tissue, as well as affecting the heart and brain. Furthermore, short-term low-level exposure causes nausea and vomiting, decreased erythrocyte and leukocyte production, and damage to blood vessels, resulting in irregular heartbeat as well as hand and leg-pricking sensations. Arsenicosis, or chronic arsenic poisoning, causes skin pigmentation and keratosis (Martin & Griswold, 2009). Neurological disorders have all been associated to long-term exposure (Huy *et al.,* 2014). Arsenicosis may cause permanent damage to important organs of the body and cause death in extreme situations (Huy *et al.,* 2014).

Mechanism of toxicity

In arsenic biotransformation, bacteria, algae, fungi, and humans methylate toxic inorganic arsenic molecules to produce monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Jaishankar *et al*., 2014). These inorganic arsenic species (iAs) are transformed enzymatically to methylated arsenicals, the end metabolites and biomarkers of chronic arsenic exposure, during the biotransformation process (Jaishankar *et al*., 2014). Biomethylation is a detoxifying process that produces methylated inorganic arsenic suchas MMA (V) and DMA (V), which are bioindicators of chronic arsenic exposure in urine. MMA (III), on the other hand, is not eliminated and stays as an intermediate product inside the cell (Jaishankar *et al*., 2014). This is one of the mechanisms involved in the generation of free radical by arsenic.

iAs(V) → iAs(III) → MMA(V) → MMA(III) → DMA(V)

2.3.10.4. Arsenic and Autism Spectrum Disorders

It has previously been stated that arsenic has capacity to directly damage a developing fetus' brain and nervous system by crossing the blood-brain barrier (Tolins *et al*.,2014). As a result, it is being proposed that arsenic could be involved in the neurodevelopmental toxicity reported in ASD children. Prenatal and infantile arsenic exposures have also been associated to learning and memory deficits, as well as a decrease in the neuron’s numbers and weight of the brain, as well as changes in

neurotransmission, *in vitro* and in animal models (Martinez *et al.,* 2008).

Similarly, previous research have found important links between arsenic exposure and ASD in children. Some case-control studies looked at the concentration of arsenic in children diagnosed with ASD. Four of these studies found elevated hair arsenic concentration in ASD children than in controls (Al-Ayadhi, 2005), while one study found lower hair arsenic levels in ASD children compared to controls. Another study was reported to find increased plasma arsenic level in ASD children relative to the controls. A similar result pattern was reported in blood and urinary arsenic level in ASD subjects and controls, while another study reported a lower blood arsenic level in ASD relative to controls (Rahbar *et al.*, 2012).

2.4. Neurotransmitters

Neurotransmitters are chemical substances released from nerve cells and serve as messengers between neurons and many other cells in the body, distributing, enhancing, and regulating signals or impulses. Heart rate, sleep, appetite, mood, and anxiety are only a few of the physical and psychological functions that can be influenced by neurotransmitters. Neurotransmitter molecules are actively at work in human brains, regulating breathing, heartbeat, learning process and attention span (Boto & Tomchik 2019; Cherry, 2019).

2.4.1. Classification of Neurotransmitters

Classification of neurotransmitters is based on the functions perform in the body. Based on this, they may be classified as follows:

1. Excitatory neurotransmitters- These have excitatory effects on neurons, which means they make it more likely for neurons to fire action potentials. Epinephrine and norepinephrine are two of the most powerful excitatory neurotransmitters.

2. Inhibitory neurotransmitters: These have inhibitory effects on neurons, which means they make it less likely for them to fire action potentials. Serotonin and gamma-aminobutyric acid are two of the most important inhibitory neurotransmitters (GABA).

3. Modulatory neurotransmitters: These are also known as neuromodulators, and they have capacity to simultaneously trigger a large range of neurons. They have a

major effect on chemical messengers. Neuromodulators are slower-acting but are triggered by axon terminals and function rapidly on other receptor neurons.

The classification of neurotransmitters based on their ability stimulate (excitatory) or repress (inhibitory) an action potential is displayed in Fig. 2.25

2.4.2. Types of Neurotransmitters

Neurotransmitters can be classified and categorize in a range of ways. They can be categorized into amino acids, peptides and monoamines in some cases (Valenzuela, 2011).

Amino Acids Types

GABA is a naturally occurring amino acid that acts as an inhibitory chemical in the body (Lindsey, 2015). GABA contributes greatly to anxiety management as well as vision and motor control.

Glutamate: This is a neurotransmitter available abundantly in the nervous system and involves in cognitive functions like memory. Excess glutamate has been linked to excitotoxicity, which can lead to cell death (Wang & Raddy, 2017). Excitotoxicity induced glutamate and cause its build-up. This has been associated to some diseases and disorders like epileptic seizures and brain injuries (Wang & Raddy, 2017).

2.4.3. Mode of Action of Neurotransmitters

Following the activation of voltage-gated Ca^{2+} channels, the presynaptic terminal releases neurotransmitters (Südhof, 2012). Conformational modifications occur as a result of the influx of Ca^{2+} ions. This caused the vesicle and plasma membrane to fuse, allowing neurotransmitters release at the synaptic cleft end. Neurotransmitters diffuse through the synaptic cleft after being released and binds a particular receptor on postsynaptic neuron membrane (Südhof, 2012). The neurotransmitter is released based on action potential, which then sends a message to its target. Neurotransmitter may either diffuseout of the synaptic cleft or be metabolized by enzymes within the synaptic cleft after it has completed its function (Südhof, 2012; Boto & Tomchik 2019). Calcium (Ca^{2+}) is essential for the release of neurotransmitters. Neurotransmitter release is hindered when Ca^{2+} channels are blocked (Boto & Tomchik 2019). This underscores the importance of Ca^{2+} homeostasis in neurotransmission.

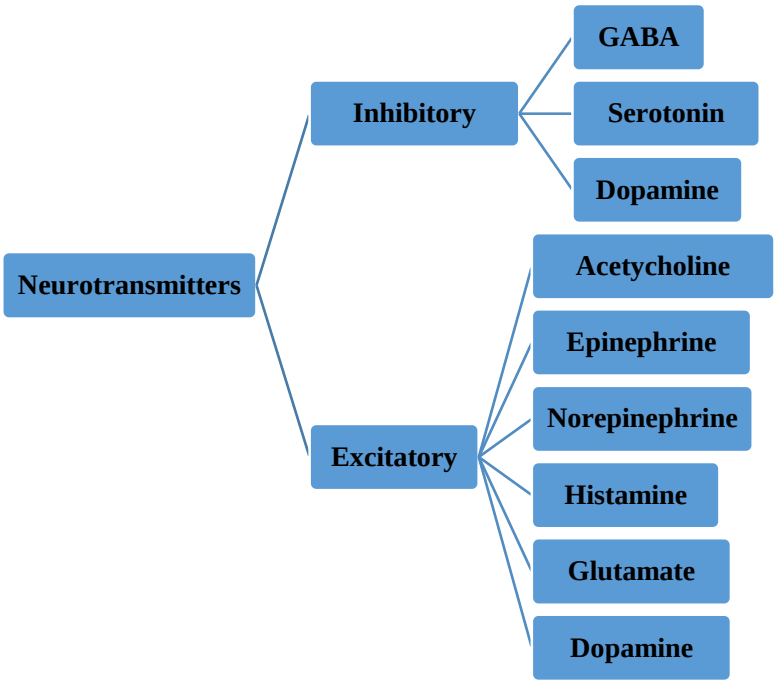


Figure 2.25: Classification of Neurotransmitters (Kumar, P., Abed, S.N., Bataineh, Y.A., Salem, M.S., 2020)

2.4.4. Neurotransmitters and Autism Spectrum Disorders

Neurotransmitters play a crucial function in brain growth, memory, motor function, and behavior control (Mittal *et al*., 2017; Choudhury *et al.,* 2018). The neurotransmitter system's malfunction is thought to facilitate neuronal cell proliferation, differentiation, and synaptogenesis, as well as the brain's developmental processes, possibly leading to ASD (Chugani, 2011). Many neurotransmitter systems have been studied in the pathophysiology of ASD, and dysfunction of these systems has been linked to the disorder. The GABAergic, glutamatergic, and serotonergic neurotransmitter systems are reported to be involved in ASD development by many studies (Cetin *et al.,* 2015; Marotta *et al*., 2020). Some of the roles of these neurotransmitters in the development of ASD are shown in Fig. 2.26.

2.4.5. Glutamine

The amino acid glutamine contributes to a range of crucial biochemical and regulatory processes and most available in the body (Cruzat *et al.,* 2018). Glutamate has the widest free range of any amino acid and is one of the most widely distributed across organs. It can be synthesizedendogenously but becomes conditionally essential in physiological and pathological conditions, resulting in its high proliferation rate (Zhou *et al.,* 2014). Glutamine is a nitrogen-containing aminoacid carrier which is produced in the muscle in large amounts and is transported to the body's different organs like the stomach, liver, and kidney. Enterocytes, hepatocytes, lymphocytes, and macrophages enjoy glutamine as a respiratory fuel (Zhou *et al.,* 2014). Beside that, glutamine appears used throughout ammonia genesis and thus plays a key role in maintaining acid-base balance (Melis *et al.,* 2004).

Glutamine is known as a non-essential amino acid in normal circumstances. Nevertheless, many research has shown that plasma glutamine concentrations drop significantly during critical illness, suggesting that glutamine can turn to conditional essential amino acid, especially in catabolic disorder. Sufficient glutamine levels are released from muscle tissue in catabolic conditions, like septicaemia or serious injury. Glutamate intake in immunologic tissues and cells increases under these conditions (Ziegler *et al.,* 2003). Glutamine is crucial for many metabolic functions including protein and glutathione synthesis, energy production, maintenance of optimal antioxidant status, and immune function (Melis *et al.,* 2004). It comprises two ammonia groups, one from glutamate as a precursor and the other from free ammonia in the

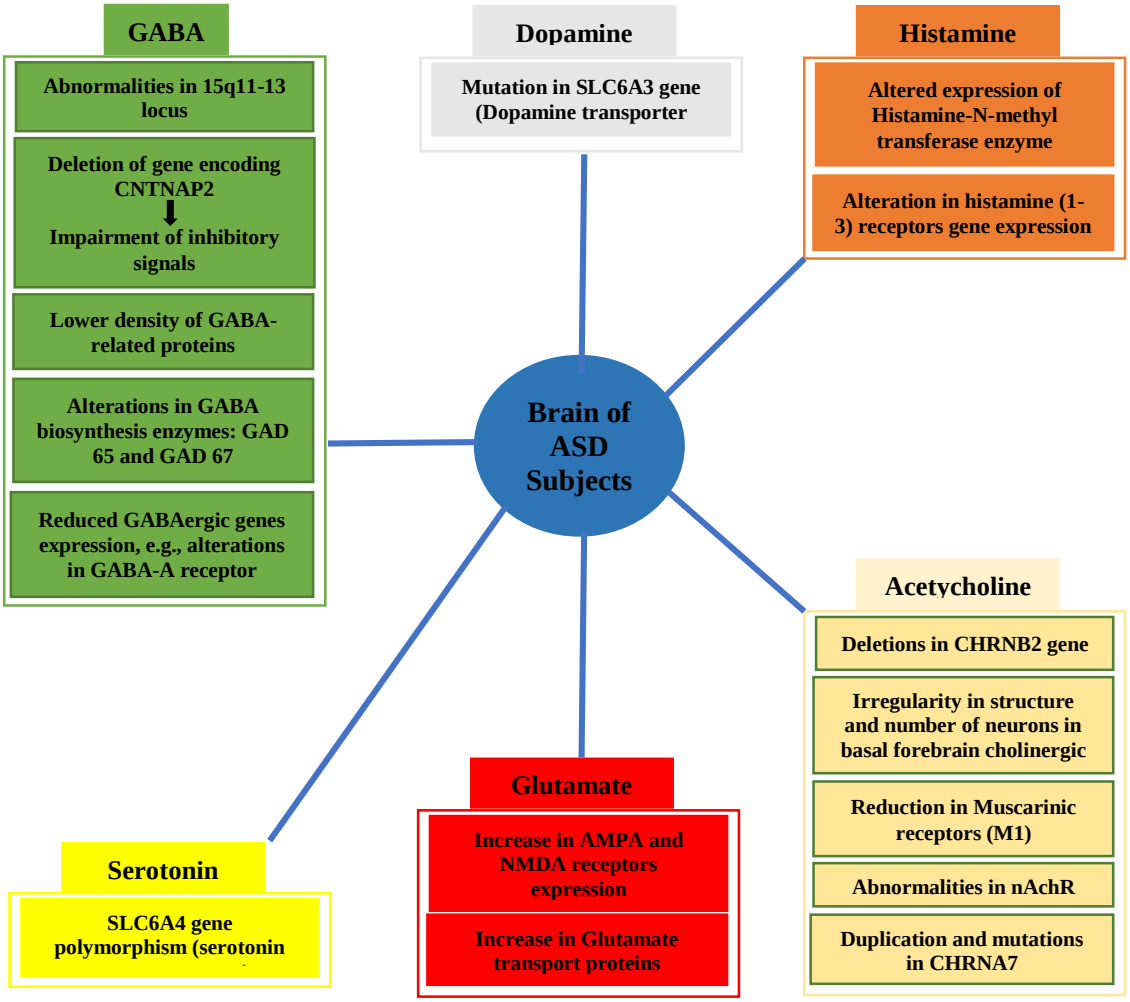


Figure 2.26: Effects of Neurotransmitters on Brain (Bronstra *et al.*, 2015).

bloodstream. Glutamine serves as a buffer, absorbing excess ammonia and releasing it when it is needed to make other amino acids, amino sugars, nucleotides, and urea, which is one of the most well-known and initial roles (Cruzat *et al.*, 2018).

2.4.5.1. Dietary sources

The dietary sources of glutamine include especially protein-rich diets from animal and plant sources. Foods such as beef, chicken, fish dairy products, sea food, nuts and legumes, eggs and beans are particularly rich sources of proteins, which are also rich natural sources of glutamine.

2.4.5.2. Absorption and distribution

Intestinal cells absorb a significant amount of dietary glutamine. However, glutamine is taken up from the circulation when the supply of glutamine from the diet is reduced. The skeletal muscle (60 percent of the total pool), stomach, brain, kidney, and liver are the major sources of glutamine. Specific organs have this amino acid for metabolic use and sufficient renal excretion. Several membrane transporters mediate glutamine uptake into cellular compartments, and control its homeostasis by facilitating its absorption, reabsorption, and delivery to tissues. Different protein families are represented by these redundant and widely distributed transporters. The role of glutamine transporters in relation to their different transport modes and coupling with Na^+ and H^+ has been identified, as well as the complex interplay between cell polarity and types of glutamine transporters. The basic transport ability of most transporters is shared with other neutral or cationic amino acids. Antiporters control glutamine and other amino acid pools, while Na^+-dependent co-transporters efficiently accumulate glutamine.

2.4.6. Glutamate

Glutamate is an amino acid that can be made in the body in large quantities. It is important for nutrition, metabolism, and signaling; it also provides an essential function in protein structure. Despite the fact that glutamate is necessary in amino acid metabolism, the majority of glutamate consumed in the diet is metabolised in the intestine (Burrin et al., 2008). Dietary glutamate has no impact on plasma glutamate levels, which is one of the disadvantages of gut glutamate metabolism. At very low levels, circulating glutamate is tightly regulated. There are two ways glutamate can be synthesized. To begin with, it can be produced by glutamate dehydrogenase or a range of amino-transferases from α-ketoglutarate. Other amino-acids such as glutamine,

arginine, proline and histidine are collectively referred to as the "glutamate family" (Plaitakis *et al*., 2017). Since the 1950s, glutamate has been known to provide an excitatory effect on mammalian brain and spinal cord generally. It was not until the late 1970s that the excitatory neurotransmitter glutamate was discovered to be the main excitatory neurotransmitter throughout the nervous system of the vertebrates (Brian, 2000).

It was also postulated that glutamate works post-synaptically on three ionotropic receptor families named after their respective agonists (Meldrum, 1998). Ionotropic glutamate receptors contain cation-permeable ion channels, but based on the receptor's group and subunit composition, relative permeability to Na^+ and Ca^{2+} differs. Molecular genetics research eventually revealed three multimeric receptors with each having subunits that are highly similar in sequence to ionotropic receptor (Zhou & Danbolt, 2014).

2.4.6.1. Release of Glutamate

Presynaptic terminal vesicles release glutamate through a Ca^{2+}-dependent mechanism that requires Ca^{2+} channels. The level of glutamate in the vesicle is around 100 mmol/L, and when a single vesicle is released, there is an inducement of an excitatory post-synaptic potential (EPSP), which is mainly associated with glutamate receptors activation (Bailey *et al.,* 2011). A number of pre-synaptic receptors regulate glutamate release synaptically. This includes not only the glutamate metabotropic receptors of Groups II and III,but also cholinergic receptors and ϒ-aminobutyric acid (GABA)B receptors (Amiry-Moghaddam & Ottersen, 2013).

2.4.6.2. Glutamate in Neurodevelopment and Neurodegeneration

The neuronal development, migration, and survival of neurons in the developing brain are all dependent on glutamate (Rahimi-Balaei et al., 2018). This is primarily due to the facilitation of Ca^{2+} entry. Blocking NMDA receptors during pregnancy can trigger cell death of the vulnerable neurons ((Alvarez *et al.,* 2013). Neurologists are particularly interested in glutamate due to its potential role in neurodegenerative diseases. Glutamate or related substances from the exogenous origin which are obtained via the diet may act on glutamate receptors and cause damage to the brain, according to one theory (Zhou & Danbolt, 2014). Second, endogenous glutamate released by neurons can play a role in acute neurodegeneration resulting from brain injury (Zhou & Danbolt, 2014). Thirdly,

in chronic neurodegenerative diseases like Huntington Parkinson diseases, glutamate receptor activation may play a crucial role in apoptosis (Zhou & Danbolt, 2014).

Glutamate's agonist effect may be neurotoxic. The importance of various receptor groups differs depending on the neurons involved and a number of other factors. NMDA receptor activation appears to be critical for selective neuronal death after status epilepticus (Hayashi *et al.,* 1999). In a number of ways, susceptibility to excitotoxic cell death is genetically regulated. Single-gene defects can increase vulnerability (Meldrum, 1998). Ca^{2+} and Cu^{2+} can then be seen as important elements in the effectiveness of glutamate as a neurotransmitter (Brian, 2000).

2.4.7. Gamma-Aminobutyric Acid

In the adult brain, gamma-aminobutyric acid (GABA) is the dominant inhibitory neurotransmitter. GABA regulates inhibitory-excitatory levels needed for brain to function properly in adults when combined with the excitatory neurotransmitter glutamate (Xu *et al*., 2014; Wu & Sun, 2015). Two primary types of receptors are available for GABA: ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. A second form of GABA receptor known as $GABA_C$ was discovered in the central nervous system, most notably in retinas of mammals (Wu & Sun, 2015). $GABA_B$ receptors, unlike ionotropic $GABA_A$ receptors, has $GABA_{B1}$ and $GABA_{B2}$ as submits. $GABA_{B2}$ is controlled by $GABA_B$, which are located at pre and post synaptic sites (Connor *et al.,* 2011). GABA acts primarily in the adult brain through the activation of $GABA_A$ receptors that are fast hyperpolarizing (Wu & Sun, 2015).

2.4.7.1 GABA and the Developing Brain

GABAergic neurons are thought to occur early in embryonic development, while glutamatergic activity occurs much later. These were reported from an animal brain study using immunocytochemistry (Wu & Sun, 2015).

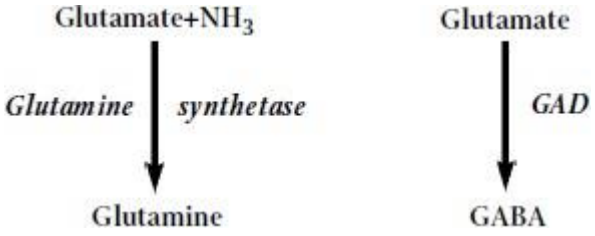
GABA is associated with most of the development effects in early embryonic, all these are facilitated by the fact that GABA acting on GABAA receptors induces an excitatory response in a developing brain; however, the developed brain later in life exhibits an opposite response, i.e., inhibitory. Evidence indicates that by controlling intracellular chloride concentration levels, cation-chloride transporters have a critical role in modulating the differential response (Hunt *et al.,* 2013). Interstitial neurons are situated

in the adult brain cortical white matter, which are grouped into two subgroups: GABAergic and glutamatergic **(**Suárez-Solá *et al*., 2009).

Anoxia-ischemia is one of the white matter lesions that may occur over time due to mechanisms involving the GABAergic system. GABA release in the white matter is reported to possess neuroprotective effect and capable of protecting neurons after anoxic injury which are expressed by compound action potential (CAP) recovery at post-anoxic (Wu & Sun, 2015).

2.4.8. Glutamate, GABA and Autism Spectrum Disorders

Many neurological functions such as cognition, perception, motion, touch and behaviour requires glutamate. Synaptic activation and its association with cell proliferation, synaptic spatial structure in the cerebellum, astrocytes and cell death are all part of the brain development. People with ASD have disrupted GABA and glutamate neurophysiology; this resulted in excitatory and inhibitory imbalance mechanisms, as reported by Rojas and colleagues in 2014. In the brain of a human, glutamate is the main excitatory neurotransmitter. Since glutamate plays such a significant and essential role, dysregulation of this neurotransmitter has also been linked to neurodevelopmental disorders and neurodegenerative disorders like ASD. Glutamate synaptic level regulation is essential to avoid glutamate accumulation, which could cause overstimulation of glutamate receptor neuronal excitotoxicity, and injury at the synaptic cleft (Choudhury *et al*., 2012).



Synaptic inhibition, on the other hand, is caused by GABA in the brain (Bjrklund, 2013). Glutamate and GABA are amino acid neurotransmitters that cannot be formed by neurons until glutamine is released into glutamatergic and GABAergic neurons from astrocytes. Glutamate is taken from synapses and transported to astrocytes, where it would be converted to glutamine and then reused. The glutamate-glutamine cycle, as well as the balance in excitatory and inhibitory neurotransmission are necessary in brain development and to avoid excitotoxicity. The process is interrupted in ASD patients'

brains and a glutamate signaling discrepancy between excitation and inhibition may be a cause of ASD, according to what was reported. Occurrence of this phenomenon in children with ASD in this environment is one of the objectives of this work. In a study conducted in Saudi Arabia, variations were discovered in glutamate and glutamine concentrations and were thought to be significant ASD predictors (El- Ansary *et al*., 2016). In autistic patients, the multiple regression study showed a strong connection between decreased GABA, neuro-inflammation and glutamate excitotoxicitythat affect glutamine concentration. This evidence suggests that these variables were linked to the severity of ASD, as measured by ASD severity questionnaires. Shimmura *et al.* (2011) published a study that looked at glutamine levels in people with AS. Plasmaglutamine and glutamate have been reported possible biomarkers for typical observed IQ in people with ASD. Although there have been few studies associating glutamate and glutamine with biomarkers of severe ASD, Attention-Deficit Hyperactivity Disorder (ADHD), which co-exists in severe ASD, was connected to glutamate and glutamine level impairment (Zablotsky *et al.*, 2017).

2.5. Oxidative Stress

Oxidative stress describes a situation where there is an imbalance in ratio of oxidants or free radicals' production and the supply and function of antioxidants, which results in macromolecule damage (Frustaci *et al.*, 2012; Castejon & Spaw, 2014). Any molecular entity capable of independent existence that has an unpaired electron in an atomic orbital is referred to as a free radical. Numerous radicals are extremely reactive and inherently unstable. Increased free radical productions or reduced antioxidant level or activity disrupt this balance. If reactive oxygen species are not quenched, this could result in oxidative damage of proteins, lipids, and DNA structure (Lobo *et al.*, 2010). Figure 2.27 depicts how oxidative stress and a host of other factors contribute to the pathogenesis of ASD.

Owing to brain's high lipid content, oxygen usage, high energy demand, and low antioxidant ability, the organ is most prone to oxidative harm (Rossignol *et al*., 2012; Essa *et al*., 2013). Phospholipid's content of the brain especially makes it more susceptible to peroxidation that is ROS- mediated; ROS also targets proteins and DNA (Salim, 2017). ROS accumulation, which is a cellular hazard, can cause substantial neuronal damage if it exceeds or bypasses counteracting mechanisms. To combat the danger posed by ROS, the brain has defensive mechanisms which are the antioxidant and

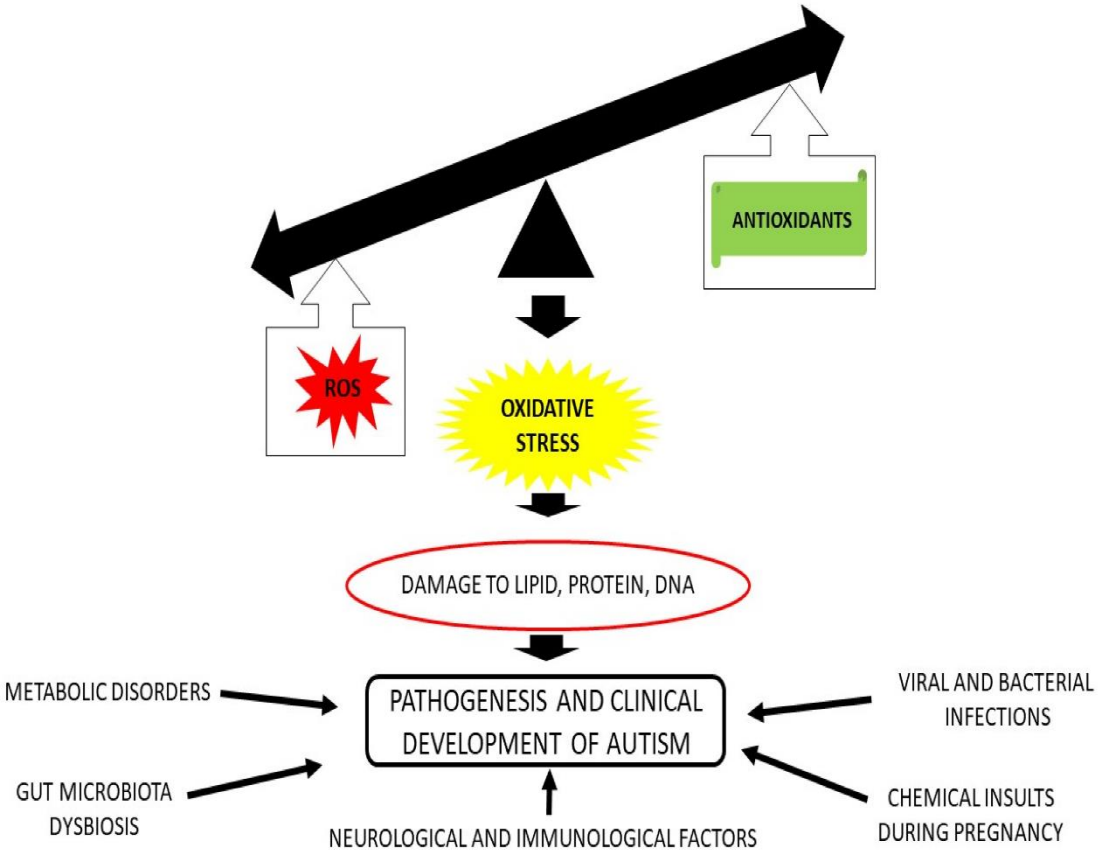


Figure 2.27: Oxidative Stress and Pathogenesis of Autism Spectrum Disorders (Membrino *et al*., 2023).

its enzyme systems. The latter include glutathione reductases, superoxide dismutases and glutathione peroxidases (Salim, 2017). The mechanism of oxidative stress as it affects the brain resulting in neurodevelopmental disorders particularly ASD is represented in Figure 2.28.

2.5.1. Markers of oxidative stress

All researchers studying the function of free radical damage in disease encounter the difficulty of accurately assessing oxidative stress in biological systems (Koracevic *et al.*, 2001). Oxidative stress can be evaluated indirectly by measuring the levels of DNA/RNA damage, lipid peroxidation, and protein oxidation/nitration than measuring reactive oxygen species directly. These oxidative stress indicators last longer than reactive oxygen species.

2.5.1.1. Lipid peroxidation

In general, lipid peroxidation is a process in which oxidants like free radicals or nonradical species attack lipids that contain carbon-carbon double bonds, particularly polyunsaturated fatty acids (PUFAs), which involve hydrogen abstraction from a carbon. Oxygen insertion results in the formation of lipid peroxyl radicals and hydroperoxides (Yin *et al.,* 2011). Enzymes like lipoxygenases, cyclooxygenases, and cytochrome P450 can also oxidize lipids in response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities which can either promote cell survival or induce cell death (Ayala *et al.*, 2014).

As demonstrated in Fig. 2.29, the process of lipid peroxidation occurs in three stages: initiation, the propagation, and termination (Yin et al., 2011). Prooxidants like the hydroxyl radical abstract the allylic hydrogen during the lipid peroxidation beginning stage to create the carbon-centered lipid radical (L•). Lipid radical (L•) quickly combines with oxygen in the propagation phase to create lipid peroxy radical (LOO•), which then takes a hydrogen atom from a different lipid molecule to create a new L• (which continues the chain reaction) and lipid hydroperoxide. (LOOH). In the termination reaction, anti-oxidants like vitamin E give a hydrogen atom to the LOO• species and create a matching vitamin E radical that combines with another LOO• to produce nonradical compounds.

Lipid hydroperoxides are the main principal byproducts of lipid peroxidation. (LOOH).

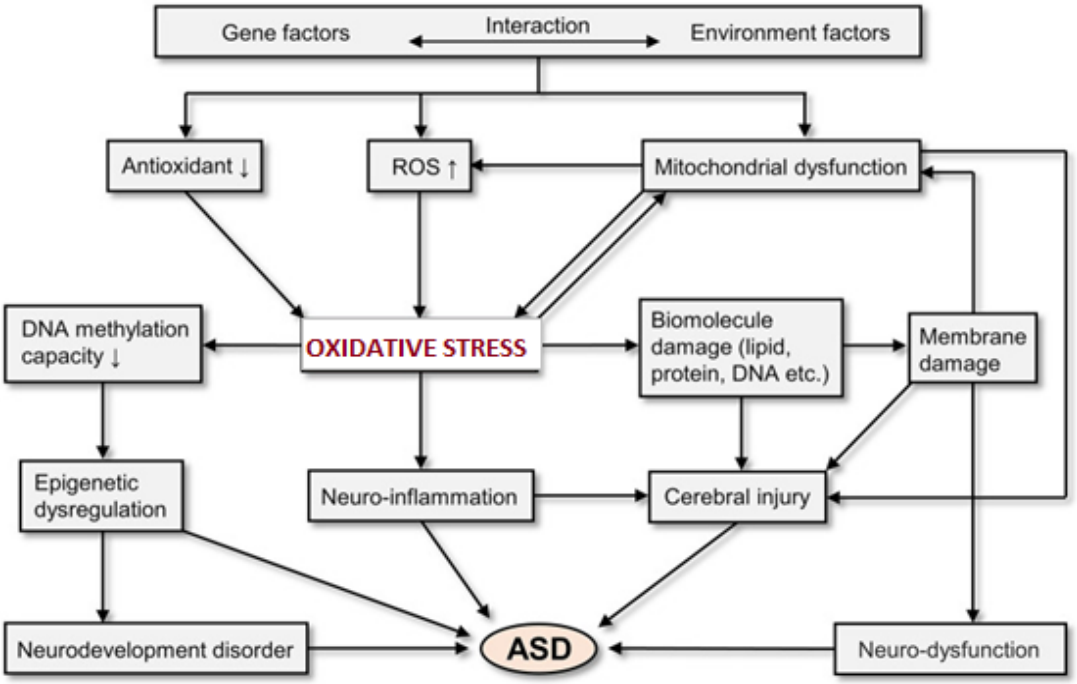


Figure 2.28: The potential mechanisms of oxidative stress in the brain of Autism Spectrum Disorders patients (Liu *et al.*, 2022).

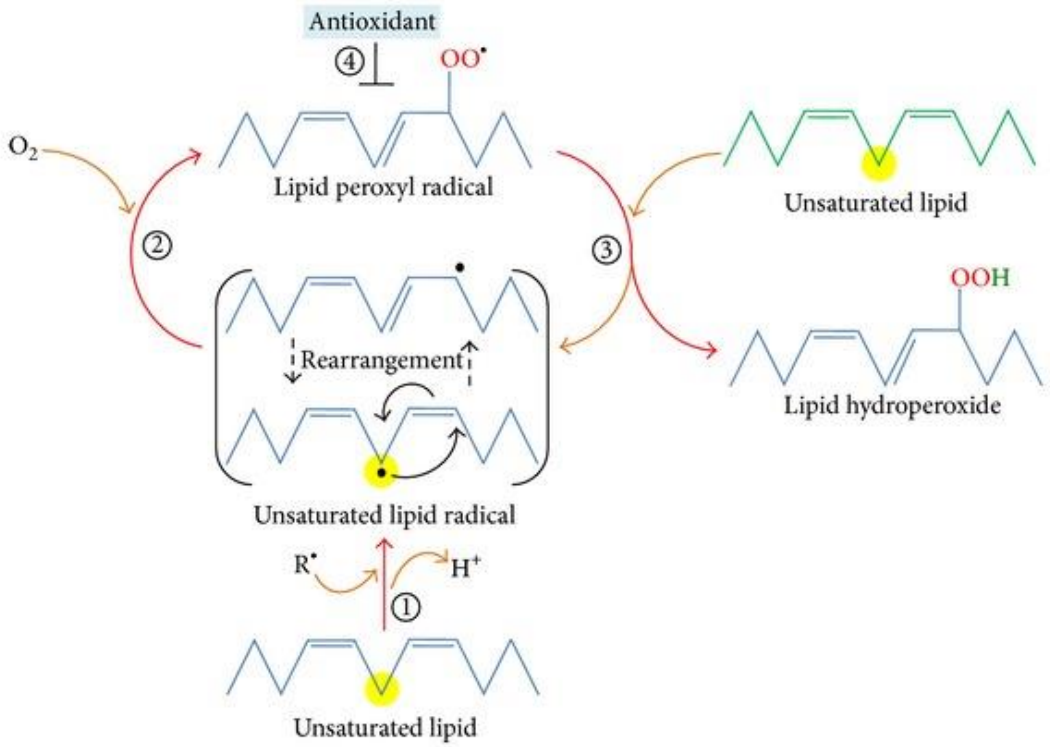


Figure 2.29: Lipid Peroxidation Process (Ayala *et al*., 2014).

Malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) are among the many distinct aldehydes that can be produced as secondary products during lipid peroxidation. Esterbauer and his colleagues conducted considerable research on these compounds in the 1980s (Esterbauer et al., 1991; Yin et al., 2011). While 4-HNE is the most lethal byproduct of lipid peroxidation, MDA seems to be the most mutagenic. MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with thiobarbituric acid (TBA). Malondialdehyde (MDA) is the most commonly used lipid marker of oxidative stress. It is formed via peroxidation of polyunsaturated fatty acids and is typically quantified using the TBARS assay.

2.5.1.2. DNA and RNA damage

As oxidative stress markers, many forms of DNA/RNA damage can be identified. The most widely used DNA damage indicator for oxidative stress is probably 8-hydroxydeoxyguanosine (8-OHdG). Less direct methods for assessing DNA damage that may be connected to oxidative stress include comet assays, tests for apurinic/apyrimidinic sites, and assays for damage caused by aldehyde.

2.5.1.3. Protein Oxidation/Nitration

Protein carbonylation and protein nitration (3-nitrotyrosines) are two examples of oxidative damage to proteins. Advanced glycation end products (AGE) and advanced oxidation protein products (AOPP) can both be produced as a result of reactive oxygenspecies. Standard assays can be used to measure each of these indicators.

2.5.1.4. Total Antioxidant Capacity

Total Antioxidant capacity (TAC) provides an integrated measurement rather than the mere total of measured antioxidants by taking into account the cumulative activity of all antioxidants presents in plasma and bodily fluids. The ability of known and unknown antioxidants is therefore evaluated, providing insight into the delicate balance between oxidants and antioxidants *in vivo*. The assessment of physiological, environmental, and dietary aspects affecting a person's redox status may be aided by measuring plasma TAC. Identifying factors influencing oxidative state *in vivo*, such as exposure to reactive oxygen species and antioxidant supplementation, may be made easier by measuring plasma TAC (Ghiselli et al., 2000). *In vivo* oxidative stress changes may not be visible

through the measurement of a single, "specific" antioxidant, but TAC is a sensitive and accurate marker to identify these changes. When the outcomes are expressed as change with respect to the basal value, the method can be used to assess the impact of various treatments on plasma redox status in healthy persons.

2.5.2. Oxidative Stress and Neurodevelopmental Disorders

Oxidative stress is connected to some neurological problems, like anxiety, depression and schizophrenia (Salim, 2014). Environmental toxicants such as heavy metals like Pb and pesticides were involved in autism (Palmer *et al*., 2009). They all have the potential to cause oxidative stress. It is also conceivable that autistic pathology is caused by a hereditary predisposition to oxidative stress stimuli and exposure to elevated levels of environmental pro-oxidants.

Oxidative stress was said to contribute to development of autism, and it could be the process of prenatal and perinatal involvement in contributing to the disorder's emergence (Mandic-Maravic *et al*., 2017). The process of transition from fetal to neonatal life for new born is a huge stress and involves a large amount of free radical production. Although healthy newborns are able to adapt to changes in oxygen concentration, problems can arise when intrauterine development is disrupted in any way (Negi *et al*., 2014). Possible intrauterine transfer of toxicants from mothers to the fetus has been reported (Punshon *et al*., 2016); however, the possibility of this leading to abnormal neurodevelopment in infancy is one of the major medical challenges in the ASD. Many studies reported that ASD patients show excessive ROS production, and the etiology of the ROS has been suggested to be both genetically and epigenetically related (Pizzino *et al*., 2017).

The key factor in the gene-environment relationship in ASD and CP is oxidative stress, according to experts (Rahbar *et al*., 2015). It was reported that there is a connection between ASD risk and Glutathione S-Transferase polymorphisms alone or together with environmental and genetic factors (Frustaci *et al*., 2012). Overstimulation of excitatory receptors causes oxidative neuronal damage, while increased oxidative stress increases glutamate release and subsequent excitatory receptor stimulation. As a result, elevated oxidative stress in ASD may indicate issues with power generation and excitotoxicity (Woody, 2004), although it is also reported to be due to lipid peroxidation in CP (Aycicek & Iscan, 2006). In ASD research, the latest trend is to look at genetic and environmental

impacts in the pathophysiology of the disorders. However, the environmental impact is one of the major focuses of this study. Previous work reported decreased total antioxidant capacity and increased lipid hydroperoxide in children with CP compared to children without CP (Ali & Akin, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Participants' Selection and Design of study

The extrapolatory case-control study was conceived to determine possible transfer of toxic and trace elements through the placenta to the baby *in-utero* and the possible link of these elements in the pathophysiology of neurodevelopmental disorders (ASD and CP) in children. Thus, two subject groups of participants were recruited using convenient sampling in the work.

Group 1: A total of one hundred and five (105) pregnant women in their third trimester with average age of 28.2±6 years were recruited for this study, and their anthropometric, demographic, medical history and environmental risk data were collected using structured questionnaires. Fifty (50) of these participants were exposed occupationally to various elements in their various vocations, and were considered case subjects. The remaining fifty-five (55) were not occupationally exposed to various elements, and these represent controls. All participants were monitored to delivery, and cord blood was collected from each of them.

Group 2: This study enlisted the participation of seventy-five (75) children. These included twenty-five (25) ASD and CP children with mean ages of 5.96±1.45 and 5.12±3.03 years, respectively, as cases and another twenty-five (25) neurotypical (NT) children with mean age of 6.18±2.59 years as controls. The controls include apparently healthy, neurotypical children drawn from a pool of relatives, colleagues, and neighbours.

Participants of groups 1 and 2 in this study received regular antenatal and childhood vaccines, respectively.

Ethical approvals were gotten from the UI/UCH joint Ethical Committee and Oyo State Ministry of Health Ethical Board. Informed consent was obtained from all participant in group 1 and through parents for group 2, and Bioethical Research Committee approval was obtained.

3.1.1 Exclusion Criteria

Pregnant women below age 20 and above 35 years as well as those suffering from diabetes and hypertension during pregnancy were excluded in category 1, while participants with internal organs dysfunction, anemia and other neurological disorders were excluded in category 2. Participants that were taking herbal supplements and concoction (*agbo*) were excluded in both categories.

3.1.2 Inclusion Criteria

Participants that gave consent directly in group 1 and by proxy in group 2 and children clinically diagnosed with neurodevelopmental disorders (ASD and CP) by child neurologists and child psychiatrists.

3.2 Diagnosis Of Neurodevelopmental Disorders

Many of the children in the research had their histories taken, which included information regarding their parents' socioeconomic status, prenatal, perinatal and postnatal history as well as their environmental exposure. Childhood illnesses at birth and immunizations history were obtained. The clinical assessment and diagnosis of all the cases with an emphasis on the neurological examination were done by consultant's neurologists and pediatric neuro-psychiatrist. All participants were assessedon a full clinical child psychiatric evaluation for inclusion and exclusion into this study (APA, 2000).

3.3 Collection Of Blood For Analysis

A venous blood sample of around 10mL was taken from ante-cubital vein of all participants. To prevent haemolysis and ensure sample viability, lithium heparin tube was used. Blood sample was centrifuged using a Centeur 2-centrifuge (MSE, UK) at 3000 rpm for 10 minutes and plasma was separated from cells into plain bottles using a Pasteur pipette.Each sample was divided into three parts and held frozen at -80°C. One group was analyzed for essential metals (Ca, Mg, Zn, Cu, Se, Mn and V) and non-essential toxic elements (Pb, Al, As). Another group of the sample was analyzed for Glutamine, Glutamate and GABA while the third group was analyzed for markers of oxidative stress (TAC, TPP, MDA and OSI). The study was a longitudinal/case-control study.

Sample Size Determination

$$N = \frac{PQZ^2}{d^2} \qquad \ldots 3.1$$

Where N is the minimum sample size desirable

Z is the confidence interval (1.96)

P = Prevalence, = 1.47% or 0.0147(CDC, 2014)

Q = 1- P = 1- 0.05 = 0.95

d = level of significance = 0.05

$$N = \frac{0.0147x0.95x1.96^2}{0.05^2}$$

$$N = \quad 21.9$$

To allow for attrition, 25 participants were recruited per group.

3.4 Anthropometric Measurements

3.4.1 Body Weight

Material: Weighing scale (Bathroom scale Hana, the big boss model, India).

Procedure:

The weighing scale was used to measure the body weight of each subject. The body weight was measured with the subject wearing only light clothes and socks, and their pockets were cleared of any objects that could affect their actual weights. The weighing scale was standardized against a fixed weight at every five readings. The readings were recorded to the nearest kilograms and before subsequent measurements; the scale was recalibrated by ensuring that the pointer returns to zero.

3.4.2 Heights

Material: Standiometre

Procedure:

The height was measured in metres with the subjects standing feet together. The horizontal bar was brought to the subjects' vertex and the reading was taken using standiometre in the nearest metre.

3.5 Analysis of Essential and Toxic Trace Elements

Trace elements were analyzed in a laboratory in the Department of Environmental and Interdisciplinary Sciences, Texas Southern University, Houston. USA.

3.5.1 Analytical Methods and Procedures

The samples were analyzed for metals using Inductively coupled plasma mass spectrometry (ICP-MS) and Inductively Coupled Plasma Optical Emission spectroscopy (ICP-OES)

Principle: This technology incorporates the use of an ICP with an MS to produce ions for elemental analysis. The ICP is involved in the development of a 10,000°C high-temperature plasma source through which the pre-treated sample was passed. At high temperature, the elements got ionized and guided further into the MS, which sorts ions based on mass/charge ratio before passing to an electron multiplier tube detector, which Identified and quantified each ion.

Procedure:

The sample was digested in a microwave digestion apparatus using concentrated nitric acid; the digested sample was diluted, fortified with internal standards and analyzed by ICP-MS.

Instrumentation

See appendix for details on instrumentation.

Calculations / identification

Instrument software was programmed to perform all necessary calculations. The results were reported in parts per billion (ppb), which was converted to SI units.

3.5.2. Estimation of Neurotransmitters

The group 2 samples were analyzed for glutamine, glutamate and GABA using MelsinHuman ELISA KIT

Principle of Method: The kit uses one-step process of double-antibody sandwich enzyme-linked immunosorbent.

Assay Procedure:

50µl of each standard was added to standard wells while 10µl of samples and 40µl sample diluent were added to the sample wells. 100µl of HRP-conjugate reagent was added to each well. The wells were covered with an adhesive strip and incubated at 37°C for 60 minutes. The wells were aspirated and washed with 400µl Wash Solution using auto washer after which the plate was inverted and blotted against clean paper towels. 50µl of each chromogen A and B were added to each well, gently mixed and incubated for 15 minutes at 37°C and Protected from light. 50µl Stop Solution was added to stop the reaction and the colour changed from blue to yellow. The intensity of the colour was read at 450 nm using a microtiter plate reader within 15 minutes.

3.5.3. Calculation of Results

The concentrations in samples were gotten from the graph.Details of reagents and materials are in Appendix

3.6 Estimation of Oxidative Stress Markers

3.6.1. Malondialdehyde (MDA) Concentration

The method used of estimating MDA was Adam-Vizi and Seregi developed in 1982. **Principle:** This method is based on the reaction between 2-thiobabituric (TBA) and MDA. On heating in an acidic medium, a pink complex colour formed was read at 532nm, the absorbance being directly proportional to the concentration of MDA in the sample (Jose, 1997).

Assay Procedure:

0.1ml of sample was added to 0.9ml of distilled water. 1.6 ml of Tris-buffer and 0.5 of 30% TCA were dispensed into the test tubes and 0.4 ml of the diluted sample was added appropriately to the test tubes. 0.5 ml of 0.75% TBA was dispensed into all the test tubes. The mixture was incubated at 80^{o}c for 45 mins and then cooled in ice and then centrifuged at 3000g for 10 minutes. The absorbance of the supernatant was read at

532nm using distilled water as a blank. MDA (unit's/mg protein) was calculated as follows:

$$\frac{absorbance\ of\ sample\ x\ volume\ of\ mixture}{volume\ of\ sample\ x\ mg\ protein\ of\ each\ sample\ x\ molar\ extinction\ coefficient} \quad 3.2$$

Molar extinction co-efficient = $1.56x10^5$ M/cm

Concentrations were multiplied by the dilution factor. Details on reagents and materials are provided in Appendix

3.6.2. Determination of Total Plasma Peroxide (TPP)

The TPP level was determined by Fox-2 reagent as described by Miyazawa, (989) with minor modifications (Harma *et al.*, 2005; Adedapo *et al.*, 2014).

Principle: The Fox-2 method is based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) by various types of peroxides contained within the plasma samples to produce a colored ferric–xylenol orange complex whose absorbance was measured at 560nm. Using a solution of H_2O_2 (100mM-H_2O_2) as a standard, the total plasma peroxide content of the sample was determined as a function of the difference in absorbance between the test and blank.

Assay Procedure:

200 µl of plasma was added to 1800 µl of pre-warmed Fox-2 reagent. The solution was incubated at room temp. for 30mins. After incubation, the solution was centrifuged at 3000 rpm for 10 mins.

The absorbance of the supernatant was measured at 560nm using a spectrophotometer.

Calculation of TPP level: $\frac{Absorbance\ of\ test\ x\ Concentration\ of\ Standard\ (100mM)}{Absorbance\ of\ Standard}$ 3.3

Details of reagents and materials are available in Appendix

3.6.3. Total Antioxidant Capacity Estimation (TAC)

The Total Antioxidant Capacity (TAC) was analyzed using Ferric Reducing Antioxidant Powder (FRAP) reagent as described by Benzie and Stram (1999).

Principle: A low pH reduction of 2,4,6-tripyridyl-s-triazone (TPTZ) ferric complex is reduced to ferrous form (which has an intense-blue color). The color change was

monitored and measured at 593nm absorbance.

Assay Procedure:

100µL of plasma was added to 3ml of FRAP reagent. The initial absorbance of mixture was measured at 593nm after vortexing. The mixture was incubated in water bath at 37˚c for 4 minutes after the first absorbance was taken, and the second absorbance was measured again after the incubation.

1. Change in absorbance was taken as A2 - A1

Concentration of TAC = $\frac{Change\ in\ absorbance\ of\ sample\ x\ concentration\ of\ standard}{Change\ in\ absorbance\ of\ standard}$

3.4

Reagents and materials details are available in

Appendix

3.7 Statistical Analysis

The data collected by the use of structured questionnaire and results of biochemical analyses were analyzed using Statistical Package for Social Science version 23 (SPSS 23). The data was assessed for normal distribution and appropriate statistical test was used. The analysis done was based on data type:

Descriptive statistics: non-numerical data were expressed in percentage and frequencies, mean ± SE was used for non normal distribution data and mean ± SD was used for normal distribution data.

Analytical statistics: The Student's t test was used to compare the means of two groups, ANOVA was used to compare the means among three (ASD, CP and NT) groups and post-hoc test (multiple comparisons) was used to identify the significant pair(s). Mann Whitney-U test was used non-normal distribution data, Chi-square was used for two qualitative variables, while Fisher's exact test was used for variables less than 5 counts. 95% confidence interval was taken to be significant.

CHAPTER FOUR

RESULTS

4.1 Anthropometric data of occupationally exposed and unexposed pregnant women

The findings from this study revealed no substantial variations in pregnancy age, pregnant women' age, husbands' age, weight, height and BMI as well as in the weight, height and head circumference of the babies. The difference observed in age at 1st birth was significant ($p<0.048$) between exposed and unexposed pregnant women. The result showed that the exposed pregnant women married at a younger age and had their first child earlier than the unexposed group (Table 4.1).

4.2 Anthropogenic data of occupationally exposed and unexposed pregnant women

Table 4.2 showed the comparison of frequency distribution of environmental factors. The result from this study showed there were no significant differences in environmental exposure factors and household dust between exposed and unexposed groups.

4.3. Nutritional History and Consumption of Dietary Supplements.

Significant differences were not seen in fruits, vegetables and nutritional supplement consumptions between the exposed and unexposed groups (Table 4.3).

4.4. Educational Background and Occupational Exposure of Participants

Analysis of structured questionnaire showed that 14% of exposed pregnant women had post-secondary education, while 94% were artisans and traders. Conversely, 47.27% had secondary education, while 46% were traders amongst unexposed women participants. ($p< 0.002$; $p < 0.008$). There were notable variations in level of education and occupation in exposed and unexposed pregnant women (Table 4.4). A further analysis of their spouses' education and occupation showed that there were also significant variations in their level of education and occupation ($p<0.001$; $p<0.000$).The exposed group spouses were mainly artisans (78%) and traders (12%) compared to unexposed group spouses which are mainly professionals (32.7%) and civil servants (22.8%) (Table 4.4).

Table 4.1: Comparison of exposed and unexposed biodata variables (Mean±SEM) Using Student T-Test

Variable	Exposed (n=50)	Unexposed (n=55)	T	P
Pregnancy age (weeks)	37.6±1.2	37.6±2.00	0.106	0.916
Age (years)	27.7±5.6	28.8±5.4	-1.082	0.282
Age at 1st birth (years)	**23.7±4.5**	**25.4±4.1**	**-1.999**	**0.048***
Husband age (years)	33.4±6.5	35.6±6.4	-1.730	0.087
Weight (kg)	63.3±10.0	63.3±10.7	0.006	0.995
Height (m)	1.6±0.1	1.6±0.1	-0.032	0.975
BMI (kg/m^2)	25.1±3.9	25.1±4.0	0.014	0.989
Baby weight (kg)	[a]3.0±0.1	[a]3.6±0.6	-1.326[b]	0.185
Baby height (inches)	48.5±2.4	47.5±3.9	1.685	0.095
Head circumference (cm)	33.3±1.4	33.5±1.70	-0.563	0.575

[a]Mean ± SEM

[b]Mann Whitney-U non parametric test z-core

***Significant at p<0.05**

TABLE 4.2: Comparison of Environmental Exposure Factors Between the Two Groups Using Chi-Square

Variables	Response	Exposed (N=50)	Non-Exposed (N=55)	X^2	P value
Tarred road	Yes	37 (74.0%)	43 (78.2%)	0.252	0.615
	No	13 (26.0%)	12 (21.8%)		
Traffic level	Light	3 (6.0%)	6 (10.9%)	1.170	0.557
	Moderate	10 (20.0%)	13 (23.6%)		
	Heavy	37 (74.0%)	36 (65.5%)		
Use of insecticide	Yes	32 (64.0%)	35(63.5%)	0.001	0.969
	No	18 (36.0%)	20(36.4%)		
House painted	Yes	39 (78.0%)	38 (70.4%)	0.786	0.375
	No	11 (22.0%)	16 (29.6%)		
House paint peeling	Inside	9 (18.0%)	5 (9.1%)	2.085	0.352
	outside	30 (60.0%)	34 (61.8%)		
	Not sure	11 (22.0%)	16 (29.1%)		
Smoking member	Yes	2 (4.0%)	4 (7.3%)	0.521	0.471
	No	48 (96.0%)	51 (92.7%)		
Water source	Piped	2 (4.0%)	8 (14.5%)	1.174	0.759
	Well	34 (68.0%)	39 (70.9%)		
	Borehole	7 (14.0%)	8 (14.5%)		

***Significant at p<0.05**

TABLE 4.3: Comparison of Exposed and Unexposed Group's Nutrition and Dietary History Using Chi-Square

Variables	Responses	Exposed (N=50)	Unexposed (N=55)	X^2	P-value
Fruits and vegetables	Daily	27 (54.0%)	31 (56.4%)	2.437	0.296
	Weekly	19 (38.0%)	15 (27.3%)		
	Occasionally	4 (12.5%)	9 (16.4%)		
Nutritional supplements	Daily	2 (6.0%)	5 (9.1%)	2.677	0.444
	Weekly	5 (10.0%)	2 (3.6%)		
	Occasionally	12 (24.0%)	10 (18.2%)		
	Not taken	30 (60.0%)	38 (69.1%)		
Sea food	Daily	27 (54.0%)	39 (70.9%)	7.380	0.061
	Weekly	19 (38.0%)	11 (20.0%)		
	Occasionally	2 (4.0%)	5 (9.1%)		
	None	2 (4.0%)	0 0.0%)		
Supplement in pregnancy	Yes	29 (58.0%)	40 (72.7%)	1.468	0.226
	No	21 (42.0%)	15 (27.3%)		

***Significant at p<0.05**

TABLE 4.4: Comparison of the Exposed and Unexposed Groups' Socio-Economic Status Frequency Distributions Using Chi-Square

Variables	Responses	Exposed (N=50)	Unexposed (N=55)	X^2	P-value
Occupation	Artisan	22 (44%)	5 (9.1%)	87.652	**0.001***
	Trading	25 (50%)	22 (40.0%)		
	Civil servant	3 (6.0%)	6 (10.9%)		
	Professional	0 (0.0%)	14 (25.45%)		
	Student	0 (0.0%)	1 (1.82%)		
	Others	0 (0.0%)	7 (12.7%)		
Husband's occupation	Artisan	39 (78.0%)	10 (18.2%)	97.555	**0.001***
	Trading	6 (12.0%)	10 (18.2%)		
	Civil servant	5 (10.0%)	12 (21.8%)		
	Professional	0 (0.0%)	18 (32.7%)		
	Student	0 (0.0%)	3 (5.5%)		
	Others	0 (0.0%)	2 (3.6%)		
Level of education	NFE	1 (2.0%)	0 (0.0%)	14.440	**0.002***
	PE	8 (16.0%)	4 (7.3%)		
	PPE	34 (68.0%)	25 (45.5%)		
	PSE	7 (14%)	26 (5.6%)		
Husband's level of education	NFE	1 (2.0%)	0 (0.0%)	19.515	**0.001***
	PE	4 (8.0%)	2 (3.6%)		
	PPE	35 (70.0%)	19 (34.5%)		
	PSE	10 (20.0%)	33 (60.0%)		
	PGE	0 (0.0%)	1 (1.8%)		

***Significant at p<0.05**
NFE: No Formal Education
PE: Primary Education
PPE: Post-Primary Education
PSE: Post-Secondary Education
PGE: Post Graduate Education

4.5 Trace and Toxic Element Levels in Pregnant Mothers Blood and Cord Blood

Toxic metals (Pb, Cd) levels in occupationally exposed pregnant mothers were higher when compared to levels in unexposed pregnant mothers; however, the differences were not significant. Also, levels of essential elements (Se, Zn, Ca, Mg, and Cu) between the cases and control were not statistically different, although the levels of Cu and Zn were lower, while that of Mg and Se were higher in the exposed group compared to the unexposed group. The level of Ca was comparable in both groups (Table 4.5).

The cord blood from exposed and unexposed groups showed no significant differences in the concentration of essential elements (Se, Zn, Ca and Cu). Meanwhile, the toxic metals (Pb, Cd) were not detectable in over 90% of the cord blood. The Cord Cu concentration was increased while that of Se was decreased in exposed group contrary to what was found in maternal blood. The Mg levels in cord blood of the exposed group was significantly reduced when compared with the unexposed group. ($p < 0.013$) (Table 4.5).

4.6 Correlation Analysis of Essential Elements in Exposed Maternal and Cord Blood

Table 4.6 shows correlations between levels of toxic and essential elements in exposed maternal and cord blood. There was a negative correlation in maternal Ca and cord blood Se in the exposed group (-0.349, 0.022*).

4.7 Correlation Analysis of Essential Elements in Unexposed Maternal and Cord Blood

Table 4.7 displays a positive correlation existed between maternal Se and cord blood Cu in the unexposed pregnant women (0.450, 0.005*) as well as direct proportionality between maternal plasma Zn level and cord Mg level in unexposed pregnant women (0.266, 0.045*).

Table 4.5: Comparison of Toxic and Essential Elements (Mean±SEM) in Exposed and Unexposed Pregnant Women Using Student T-Test

Variable	Exposed (n=50)	Unexposed (n=55)	T-value	P-value
Maternal Cd (µg/dl)	[a]96.7±15.6	[a]70.0±30.0	-0.519[b]	0.604
Maternal Pb (µg/dl)	11.0±1.4	10.0±1.9	1.000	0.423
Maternal Cu (µg/dl)	328.0±110.0	348.3±150.6	-0.780	0.437
Maternal Zn (µg/dl)	370.8±193.0	416.8±276.7	-0.978	0.330
Maternal Ca (mg/dL)	8.6±0.9	8.6±0.9	0.034	0.973
Maternal Mg (mg/dL)	1.5±0.3	1.5±0.4	1.088	0.279
Maternal Se (µg/dl)	[a]10.2±1.2	[a]9.0±1.2	-0.528[b]	0.597
Cord Cu (µg/dl)	[a]125.1±24.7	[a]91.1±13.3	-0.807[b]	0.420
Cord Zn	[a]525.4±45.9	[a]591.2±44.6	-1.028[b]	0.306
Cord Ca (mg/dL)	8.4±0.2	8.5±0.1	-0.291	0.772
Cord Mg (mg/dL)	**[a]1.5±0.3**	**[a]1.6±0.2**	**-2.531**	**0.013***
Cord Se (µg/dl)	[a]7.0±0.7	[a]8.2±0.8	-1.235[b]	0.217

***Significant at p<0.05**

[a]Mean ±SEM

[b]Mann Whitney-U non parametric test z-core

Table 4.6: Correlation Between the Levels of Toxic and Essential Elements in the Blood of Exposed Pregnant Women and their Cord Blood

Maternal variables	Cu (r, p)	Zn (r, p)	Ca (r, p)	Mg (r, p)	Se (r, p)
Baby weight	-0.073, 0.613	-0.148, 0.304	-0.196, 0.173	0.072, 0.617	-0.098, 0.521
Baby height	0.014, 0.921	-0.091, 0.532	-0.036, 0.804	0.033, 0.822	-0.004, 0.977
Head circumference	-0.090, 0.533	-0.013, 0.928	-0.011, 0.941	0.080, 0.580	-0.022, 0.884
Cord Cu	0.030, 0.881	0.213, 0.276	-0.032, 0.873	0.247, 0.204	0.041, 0.840
Cord Zn	-0.041, 0.775	-0.015, 0.916	-0.105, 0.468	-0.035, 0.808	-0.082, 0.591
Cord Ca	-0.007, 0.962	-0.044, 0.759	0.204, 0.156	0.092, 0.526	-0.090, 0.555
Cord Mg	0.264, 0.064	0.274, 0.054	-0.107, 0.462	0.263, 0.065	-0.025, 0.868
Cord Se	-0.105, 0.502	-0.089, 0.570	**-0.349, 0.022***	0.149, 0.340	-0.017, 0.920

***Significant at p<0.05**

Table 4.7: Correlation Between the levels of Toxic and Essential Elements in the Blood of Unexposed Pregnant Women and their Cord Blood

Maternal Variables	Cu (r, p)	Zn (r, p)	Ca (r, p)	Mg (r, p)	Se (r, p)
Baby weight	0.049, 0.725	0.070, 0.611	-0.162, 0.236	0.096, 0.484	-0.150, 0.288
Baby height	0.130, 0.344	0.253, 0.063	0.118, 0.391	0.101, 0.462	0.049, 0.729
Head circumference	0.057, 0.679	0.073, 0.597	-0.022, 0.873	0.052, 0.704	-0.023, 0.871
Cord Cu	-0.034, 0.832	0.109, 0.497	0.087, 0.587	0.123, 0.443	**0.450, 0.005***
Cord Zn	-0.131, 0.341	0.163, 0.235	-0.232, 0.088	-0.187, 0.171	0.177, 0.210
Cord Ca	-0.028, 0.838	0.018, 0.895	0.093, 0.500	0.200, 0.143	-0.105, 0.460
Cord Mg	0.136, 0.321	**0.266, 0.045***	-0.130, 0.345	0.007, 0.958	0.018, 0.900
Cord Se	0.114, 0.411	0.058, 0.677	-0.134, 0.335	0.045, 0.745	-0.060, 0.677

***Significant at p<0.05**

4.8 Comparative Analysis of Biodata Variables of ASD, CP and NT Children

Table 4.8 summarises the comparison of biodata variable across the three categories (ASD, CP and NT) of participants in group II of this study. Among the three groups, there were no major variations in infant age, maternal age at child's birth, paternal age at child's birth, or child's weight ($p<0.022$); notwithstanding, there was parity between cases and control.

4.9 Gender Distribution in the ASD, CP and NT Groups

Table 4.9 shows the frequency distribution of family history within the groups. There was a preponderance ratio of 5:1 (male to female) in ASD and 3:1 for CP with an odd ratio of 68% for ASD and 52% for CP for being the first child among siblings.

4.10 Socio-Economic Status of Maternal and Paternal Participants

The comparison of socio-economic status within the group was shown in Table 4.10. The maternal and paternal levels of education **(**$p<0.002$; $p<0.000$) and occupation ($p<0.001$; $p<0.002$) were significantly different between the groups. The CP children maternal and paternal occupations were mainly trading and artisan respectively while ASD and NT maternal and paternal occupations civil service. The parents of CP children were less educated compared to ASD and NT.

4.11. Comparison of Developmental Milestones among ASD, CP and NT Groups

The frequency distribution of developmental milestones is shown in Table 4.11. When comparing between the groups, the study found 28 % of ASD participants and 64% of CP participants had speech abnormality ($p < 0.001$). Of the developmental milestones' indicators, CP participants were significantly affected relative to the NT and ASD children. Also, 28 % of children with CP had an unstable neck and delayed sitting ($p < 0.001$), while 36 % were not crawling and 52 % were not walking($p<0.001$). The onset of these developmental milestones showed significant differencesin stable neck ($p < 0.001$), sitting ($p < 0.001$), crawling ($p < 0.001$), walking ($p < 0.001$) andtalking ($p<0.001$) between ASD, CP and NT groups.

Table 4.8: Analysis Of Biodata Variables Distributions Among ASD, CP and NT Groups Using ANOVA. (Mean ± SD) (multiple comparisons)

Variables	ASD (N=25)	CP (N=25)	NT (N=25)	F-value	P-value
Child's age (yrs.)	5.96±1.45	5.12±3.03	6.18±2.59	1.303	0.278
Maternal age at child's birth (yrs.)	26.68±2.69	27.76±4.88	27.96±2.70	0.929	0.400
Paternal age at child's birth (yrs.)	31.72±2.98	33.28±6.52	32.32±3.86	0.700	0.500
Number of child's siblings	1.72±0.79	1.64±1.19	1.60±0.91	0.098	0.907
Child's weight (kg)	19.64±3.99	16.94±8.07	19.00±5.18	1.381	0.258
Child's birth weight (kg)	3.35±0.28[b]	2.90±0.73	3.03±0.63	4.032	**0.022***

***Significant at p<0.05**

Table 4.9: The Frequency Distribution of Family History Among ASD, CP and NT Groups Using Chi-Square

Variables	Response	ASD (N=25)	CP (N=25)	NT (N=25)	X^2	P-value
Gender	Male	21 (84.0)	19 (76.0)	17 (68.0)	1.754	0.416
	Female	4 (16.0)	6 (24.0)	8 (32.0)		
State of origin	North	1 (4.0)	0 (0.0)	0 (0.0)	9.969	0.126
	South	0 (0.0)	1 (4.0)	0 (0.0)		
	East	8 (32.0)	2 (8.0)	3 (12.0)		
	West	16 (64.0)	22 (88.0)	22 (88.0)		
Place of residence	North	2 (8.0)	0 (0.0)	0 (0.0)	6.250	0.181
	South	0 (0.0)	0 (0.0)	0 (0.0)		
	East	1 (4.0)	0 (0.0)	0 (0.0)		
	West	22 (88.0)	25 (100.0)	25 (100.0)		
Child birth order	1st	17 (68.0)	13 (52.0)	12 (48.0)	12.520	0.129
	2nd	8 (32.0)	6 (24.0)	11 (44.0)		
	3rd above	0 (0.0)	6 (24.0)	2 (8.0)		
Siblings with same condition	Yes	0 (0.0)	1 (4.0)	0 (0.0)	2.027	0.363
	No	25 (100.0)	24 (96.0)	25 (100.0)		

***Significant at p<0.05**

Table 4.10: Comparison of the Socio-Economic Status Between ASD, CP and NT Groups Using Chi-Square

Variables	Response	ASD (N=25)	CP (N=25)	NT (N=25)	X^2	P-value
Maternal occupation	Artisan	0 (0.0)	3 (12.0)	0 (0.0)	23.985	**0.001***
	Civil servant	16 (64.0)	7 (28.0)	22 (88.0)		
	Student	1 (4.0)	0 (0.0)	0 (0.0)		
	Trading	8 (32.0)	15 (60.0)	3 (12.0)		
Paternal occupation	Artisan	2 (8.0)	8 (32.0)	0 (0.0)	16.592	**0.002***
	Civil servant	18 (72.0)	11 (44.0)	23 (92.0)		
	Trading	5 (20.0)	6 (24.0)	2 (8.0)		
Maternal level of education	PE	(0.0)	4 (16.0)	0 (0.0)	21.031	**0.002***
	PPE	2 (8.0)	5 (20.0)	6 (24.0)		
	PSE	19 (76.0)	14.(56.0)	9 (36.0)		
	PGE	4 (16.0)	2 (8.0)	10 (40)		
Paternal level of education	PPE	3 (12.0)	7(28.0)	6 (24.0)	24.282	**< 0.000***
	PSE	16 (64.0)	16 (64.0)	3 (12.0)		
	PGE	6 (24.0)	2 (8.0)	16 (64.0)		

***Significant at p<0.05**
NFE: No Formal Education
PE: Primary Education
PPE: Post-Primary Education
PSE: Post-Secondary Education
PGE: Post Graduate Education

Table 4.11: Comparison of Developmental Milestone Onset Between ASD, CP and NT Children Using Chi-Square

Variables	Response	ASD (N=25)	CP (N=25)	NT (N=25)	X^2	P-value
Stable neck	Yes	25 (100.0)	18 (72.0)	25 (100.0)	15.441	**<0.001***
	No	0 (0.0)	7 (28.0)	0 (0.0)		
Sitting	Yes	25 (100.0)	18 (72.0)	25 (100.0)	15.441	**<0.001***
	No	0.(0,0)	7 (28.0)	0 (0.0)		
Crawling	Yes	25 (100.0)	16 (64.0)	25 (100.0)	20.455	**<0.001***
	No	0 (0.0)	9 (36.0)	0 (0.0)		
Walking	Yes	25 (100.0)	12 (48.0)	25 (100.0)	31.452	**<0.001***
	No	0 (0.0)	13 (52.0)	0 (0.0)		
Talking	Yes	18 (72.0)	9 (36.0)	25 (100.0)	24.206	**<0.001***
	No	7 (28.0)	16 (64.0).	0 (0.0)		

***Significant at P<0.05**

4.12. Comparison of Exposure to Environmental and Household Dust Among the Groups

Table 4.12 is a summary of comparative analysis of data on environmental exposure to environmental and household dust. The study results showed that there were significant differences in the factors except in the area of house paint peeling. The study highlighted that 68% and 17% of CP and ASD participants, respectively, have their house situated in an untarred road environment ($p < 0.001$). The level of traffic was moderate (52%) in CP ($p < 0.025$).

4.13. Comparative Analysis of Environmental Exposure to Smoke, Waste dump, Insecticide and Pica among ASD, CP and NT Groups

Table 4.13 is the summary of comparative analysis of data on environmental exposure to smoke, waste dump, insecticide and pica. The results showed no major variations in smoking, source of drinking water and use of insecticide but a significant variation was observed in factory situated around the house ($p < 0.044$), 23% of ASD and 100% CP were showed to be drinking untreated water ($p < 0.001$). 40% NT children indulged in placing toys and other objects in the mouth against 24% found in ASD and CP ($p < 0.001$).

4.14. Comparison of Fruits, Vegetables/Dietary and Supplement Consumption Between the Groups

Access and affordability of participants to fruits, vegetables, sea foods and nutritional supplements was summarized in Table 4.14. There were no major variations in consumption of fruits and vegetables, nutritional supplements, sea foods and supplements between the three groups according to the findings of this study. In other words, diet and nutritional status of the participants were similar.

Table 4.12: Comparison of Frequency of Exposure to Environmental And Household Dust Within the Groups (ASD, CP and NT) Using Chi-Square

Variables	Response	ASD (N=25)	CP (N=25)	NT (N=25)	X^2	P-value
Tarred road	Yes	21 (84.0)	8 (32.0)	22 (88.0)	22.426	**<0.001***
	No	4 (16.0)	17 (68.0)	3 (12.0)		
Traffic level	Light	17 (68.0)	10 (40.0)	20 (80.0)	11.131	**0.025***
	Moderate	8 (32.0)	13 (52.0)	5 (20.0)		
	Heavy	0 (0.0%)	2 (8.0%)	0 (0.0%)		
House painted	Yes	25 (100.0)	20 (80.0)	25 (100.0)	10.714	**0.005***
	No	0 (0.0)	5 (20.0)	0 (0.0)		
Part of house painted	Inside	4 (16.0)	3 (12.0)	7 (28.0)	16.549	**0.011***
	Outside	0 (0.0)	1 (4.0)	3 (12.0)		
	Both	21 (84.0)	16 (64.0)	15 (60.0)		
	None	0 (0.0)	5 (20.0)	0 (0.0)		
House paint peeling	Yes	3 (12.0)	5 (20.0)	5 (20.0)	0.744	0.689
	No	22 (88.0)	20 (80.0)	20 (80.0)		
Dumping site near house	Yes	3 (12.0)	1 (4.0)	3 (12.0)	1.261	0.531
	No	22 (88.0)	24 (96.0)	22 (88.0)		

***Significant at p<0.05**

Table 4.13: Comparison of Frequency of Environmental Exposure to Smoke, Waste Effluent And Dichlorophosphate (Insecticide) within the Groups (ASD, CP and NT) Using Chi-Square

Variables	Response	ASD (N=25)	CP (N=25)	NT (N=25)	X^2 Value	P-value
Smoking member	Yes	3 (12.0)	3 (12.0)	3 (12.0)	0.000	1.000
	No	22 (88.0)	22 (88.0)	22 (88.0)		
Factory situated	Yes	0 (0.0)	3 (12.0)	0 (0.0)	6.250	**0.044***
	No	25 (100.0)	22 (88.0)	25 (100.0)		
Source of Water	Borehole	3 (12.0)	3 (12.0)	4 (16.0)	2.440	0.655
	Piped	11 (44.0)	10 (40.0)	6 (24.0)		
	Well	11 (44.0)	12 (48.0)	15 (60.0)		
Water treatment	Yes	2 (8.0)	0 (0.0)	9 (36.0)	14.276	**0.001***
	No	23 (92.0)	25 (100)	9 (36.0)		
Use of insecticide	Yes	20 (80.0)	17 (68.0)	20 (80.0)	1.316	0.518
	No	5 (20.0)	8 (32.0)	5 (20.0)		
Placing object in mouth	Yes	6 (24.0)	6 (24.0)	10 (40.0)	22.191	**<0.001***
	No	19 (76.0)	19 (76,0)	15 (60.0)		

***Significant at p<0.0**

Table 4.14: Comparison of Frequency of Consumption of Fruits, Vegetables, Dietary Supplement Among the Groups (ASD, CP and NT) Using Chi-Square

Variables	Response	ASD (N=25)	CP (N=25)	NT (N=25)	X^2 value	P-value
Fruits and vegetables	None	0 (0.0)	1 (4.0)	0 (0.0)	10.320	0.112
	daily	13 (52.0)	9 (36.0)	18 (72.0)		
	weekly	8 (32.0)	12 (48.0)	7 (28.0)		
	Occasionally	4 (16.0)	3 (12.0)	0 (0.0)		
Nutritional supplements	None	13 (52.0)	14 (56.0)	15 (60.0)	8.693	0.192
	Daily	8 (32.0)	9 (36.0)	3 (12.0)		
	Weekly	1 (4.0)	0 (0.0)	4 (16.0)		
	Occasionally	3 (12.0)	2 (8.0)	3 (12.0)		
Sea food	None	4 (16.0)	2 (8.0)	1 (4.0)	9.574	0.144
	Daily	13 (52.0)	13 (52.0)	9 (36.0)		
	Weekly	2 (8.0)	0 (0.0)	5 (20.0)		
	Occasionally	6 (24.0)	10 (40.0)	10 (40.0)		
Supplements in pregnancy	Yes	25 (100.0)	25 (100.0)	25 (100.0)	0.000	1.000
	No	0 (0.0)	0 (0.0)	0 (0.0)		

***Significant at p<0.05**

4.15. Comparison of Antenatal Care of Mothers and Medical History of ASD, CP and NT participants.

Table 4.15 summarizes and compare the medical history of the three groups (ASD, CP AND NT) and antenatal care history of their mothers during pregnancy. The results showed a significant difference in health issues experienced by the mother during pregnancy, as 24% of mothers of CP children had health challenges in pregnancy of the child against 0% in ASD and NT ($p < 0.001$). Also, 84% of children with CP had one health issue or the other at birth compared to 8% and 0% of ASD and NT children, respectively ($p < 0.001$). Birth Asphyxia (60%) and respiratory distress (20%) were the leading health problems encountered at birth of children with CP ($p < 0.001$). The result also showed that 92% and 52% ASD and CP children, respectively, have haemoglobin Genotype AA. This implies that maternal and fetal health during prenatal, perinatal and postnatal stages may be involved in development of NDDs, particularly CP. Birth Asphyxia and respiratory distress may be the leading health issues contributing to the development of CP.

4.16. Comparison of Developmental Milestones Among the Groups

Analysis of data on the onset of developmental milestones in ASD, CP and NT children was summarized in Table 4.16. The results showed significant differences in stable neck ($p < 0.001$), sitting ($p < 0.001$), crawling ($p < 0.001$), walking ($p < 0.001$) and talking ($p < 0.001$) among the three groups. Children with CP had delayed onset of all the developmental milestones, while the children with ASD had a delay mainly in talking.

Table 4.15: Comparison of Antenatal and Medical History Between the Groups (ASD, CP and NT) Using Chi-Square

Variables	Response	ASD (N=25)	CP (N=25)	NT (N=25)	X^2	P-value
Normal pregnancy	Yes	25 (100.0)	19 (76.0)	25 (100.0)	13.043	**0.001***
	No	0 (0.0)	6 (24.0)	0 (0.0)		
Delivery method	Normal	13 (52.0)	18 (72.0)	19 (76.0)	3.720	0.156
	Cesarean section	12 (48.0)	7 (28.0)	6 (24.0)		
Birth health issue	Yes	2 (8.0)	21 (84.0)	0 (0.0)	50.543	**<0.001***
	No	23 (92.0)	4 (16.0)	25 (100.0)		
Health issue	None	22 (88.0)	2 (8.0)	25 (100.0)	57.555	**<0.001***
	B/Asphyxia	2 (8.0)	15 (60.0)	0 (0.0)		
	Premature	0 (0.0)	1 (4.0)	0 (0.0)		
	Resp. Distress	0 (0.0)	5 (20.0)	0 (0.0)		
	Seizure	1 (4.0)	1 (4.0)	0 (0.0)		
	Spam	0 (0.0)	1 (4.0)	0 (0.0)		
Jaundice	Yes	5 (20.0)	8 (32.0)	5 (20.0)	1.316	0.518
	No	20 (80.0)	17 (68.0)	20 (80.0)		
Use of oxygen	Yes	0 (0.0)	15 (60.0)	0 (0.0)	37.500	**<0.001***
	No	25 (100.0)	10 (40.0)	25 (100.0)		
Childhood vaccination	Yes	25 (100.0)	25 (100.0)	25 (100.0)	0.000	1.000
	No	0 (0.0)	0 (0.0)	0 (0.0)		
Vaccinatio nreaction	Yes	0 (0.0)	4 (16.0)	0 (0.0)	8.451	**0.015***
	No	25 (100.0)	21 (84.0)	25 (100.0)		
Haemoglobin genotype	AA	23 (92.0)	13 (52.0)	13 (52.0)	15.082	**0.020***
	AC	1 (4.0)	2 (8.0)	4 (12.0)		
	AS	1 (4.0)	10 (40.0)	7 (28.0)		
	SC	0 (0.0)	0 (0.0)	1 (4.0)		

***Significant at p<0.05**

Table 4.16: Comparison of Onset of Developmental Milestones Between the three Groups (ASD, CP and NT) Using ANOVA (multiple comparisons)

Variables	ASD (N=25)	CP (N=25)	NT (N=25)	F- value	P- value
Stable-neck (months)	3.76±0.97[b]	8.72±6.97[a]	3.00±0.96	14.377	**<0.001***
Sitting (months)	5.68±0.85[b]	13.00±9.31	4.82±1.22	17.117	**<0.001***
Crawling (months)	7.72±1.49[b]	13.50±8.73	6.16±1.46	13.952	**<0.001***
Walking (months)	12.76±2.99[b]	19.77±7.18	11.68±2.50	18.403	**<0.001***
Talking (months)	27.67±6.60[a,b]	25.11±27.02	13.04±3.86	8.836	**0.001***

***Significant at p<0.05**

a-**Significantly different from NT**

b-**Significantly different from CP**

4.17. Comparison of Concentration of Toxic and Essential Trace Metals Among the Groups

Table 4.17 shows the levels of toxic (Pb, Al and As) and essential elements (Mg, V, Ca, Zn, Se, Cu and Mn) concentrations among the three groups of participants. The results showed significant differences ($p < 0.001$) in levels of toxic elements, namely Pb, Al and As between the groups. Also, there were significant differences in levels of essential elements Mg ($p < 0.001$), Ca ($p < 0.001$), V ($p < 0.001$), Zn ($p < 0.001$), Se ($p < 0.001$), Mn ($p < 0.010$) and Cu ($p < 0.004$) between the groups. In the same way, Zn/Cu ratio was significantly different ($p < 0.001$) between the groups. The concentrations of Pb, Al and Mn were found to be highest in CP, followed by that of ASD and NT children. However, levels of essential elements Mg, Zn and V were found to be lowest in ASD, while concentration of Cu, Se and Ca were lowest in CP.

4.18. Comparison of Levels of Neurotransmitters and Oxidative Stress Markers Among ASD, CP and NT Children

Table 4.18 shows the difference in neurotransmitters and oxidative stress markers between the groups. Significant variations were observed in Glutamine ($p < 0.000$) and GABA ($p < 0.004$) concentrations as well as Gte/Glu ratio ($p < 0.000$) between the ASD, CP and NT groups. Glutamate level and GABA/Glutamate ratio showed no significant differences. The differences in levels of oxidative stress markers were significant for TPP ($p < 0.000$), TAC ($p < 0.000$), MDA ($p < 0.000$) and OSI ($p < 0.000$) between the three groups of the children. This study showed that levels of glutamate, GABA and MDA were highest in ASD, while TPP and OSI were highest in CP.

4.19. Comparison of Biodata Variables among the Groups of Children (ASD, CP and NT Children)

Table 4.19 shows the summary of analysis of biodata variables between children with neurodevelopmental disorders (ASD and CP) and controls (NT). There were no observed notable variations in the child's age, maternal's age at child's birth, or the paternal's age at child's birth, child's birth weight and current weight between children with NDDs and that of NT children.

Table 4.17: Comparison of the Levels of Trace Elements in ASD, CP and NT Children Using ANOVA (multiple comparisons)

Toxic/trace elements	ASD (N=25)	CP (N=25)	NT (N=25)	F-value	P-value
Pb (µg/dl)	9.49±4.04[a]	11.07±5.81[a]	5.43±2.04	11.706	**<0.001***
Al (µg/dl)	1.18±0.67[a]	1.32±0.74[a]	0.27±0.25	23.314	**<0.001***
V (µg/dl)	0.48±0.28[a]	0.49±0.25[a]	0.70±0.31	5.012	**<0.001***
Mg (mg/dl)	2.53±0.46[a,b]	2.82±0.64[a]	3.13±0.43	8.601	**< 0.001***
Ca (mg/dl)	7.91±1.38[a]	7.68±1.56[a]	9.78±1.27	16.579	**<0.001***
Zn (µg/dl)	222.3±63.8[a]	233.8±105.3[a]	438.5±185.5	22.398	**<0.001***
Se (µg/dl)	40.84±7.86[a,b]	27.62±6.76[a]	59.03±5.28	137.802	**<0.001***
Mn (µg/dl)	0.17±0.24[a]	0.21±0.20[a]	0.051±0.07	4.878	**0.010***
Cu (µg/dl)	4.32±1.02[a]	4.00±0.77[a]	4.88±0.94	5.960	**0.004***
As (µg/dl)	5.19±1.02[a]	5.06±0.96[a]	3.47±1.03	22.880	**<0.001***
Zn/Cu	55.31±22.04[a]	60.57±27.77[a]	92.29±44.57	9.258	**<0.001***

***Significant at p<0.05**

a-**Significantly different from NT**

b-**Significantly different from CP**

Table 4.18: Comparison of Neurotransmitters and Oxidative Stress Makers Among ASD, CP and NT Children Using ANOVA (multiple comparisons)

Neurotranmitters /oxidative stress markers	ASD (N=25)	CP (N=25)	NT (N=25)	F- value	P- value
Glutamine (µmol/l)	379.2±53.1[a,b]	296.3±59.6[a]	419.1±71.8	25.534	**<0.001***
Glutamate (nmol/ml)	1.88±0.18[a]	1.80±0.33	1.71±0.25	2.544	0.086
GABA (µmol/l)	2.07±0.34[a,b]	1.79±0.43	1.77±0.25	5.917	**0.004***
Gte/Glu	0.005±0.001[a,b]	0.006±0.002[a]	0.004±0.001	17.294	**<0.001***
GABA/Gte	1.11±0.18	1.01±0.24	1.06±0.26	1.146	0.324
Total Plasma Peroxidase	105.9±2.3[a,b]	115.1±8.5[a]	110.4±7.9	11.347	**<0.001***
Total Antioxidant Capacity	280.2±34.4[b]	209.8±57.9[a]	303.8±33.1	31.860	**<0.001***
Malondialdyde ($x10^{-5}$)	2.27±0.23[a,b]	2.08±0.17[a]	1.42±0.13	151.807	**<0.001***
Oxidative Stress Index	0.38±0.05[b]	0.60±0.23[a]	0.37±0.05	22.537	**<0.001***

***Significant at p<0.05**

a-Significantly different from NT

b-Significantly different from CP

Total Plasma Peroxidase- TPP

Total Antioxidant Capacity – TAC

Malondialdyde – MDA

Oxidative Stress Index – OSI

Gte/Glu- glutamate/glutamine ratio

Table 4.19: Comparison of Biodata Variables Between Children with NDDs and NT Children Using Student T- Test

Variables	NDDs (N=50)	NT (N=25)	T-value	P-value
Child's age (yrs.)	5.5±2.4	6.2±2.2.6	-1.063	0.291
Maternal's age atchild's Birth (yrs.)	27.2±3.9	28.0±2.7	-0.845	0.401
Paternal's age at child's Birth (yrs.)	32.5±5.1	32.3±3.9	0.156	0.877
Number of child's Siblings	1.7±1.0	1.6±0.9	0.336	0.738
Child's weight (kg)	18.3±6.4	19.0±5.12	-0.477	0.635
Child's birth weight (kg)	3.1±0.6	3.0±0.6	0.626	0.533

***Significant at p<0.05**

NDDs – Neuro-Developmental Disorders

NT - Neurotypical

4.20. Comparing Onset of Developmental Milestones Between Children with NDDs and NT Children

Table 4.20 displays result of comparative analysis of onset of developmental milestones between NDDs and NT children. The results revealed significant delay in stable neck ($p < 0.008$), sitting ($p < 0.007$), crawling ($p < 0.003$), walking ($p < 0.007$) and talking ($p < 0.000$) in participants with NDDs compared to NT children. This implies that developmental milestones were grossly delayed in these disorders and may be the early signs and symptoms in recognizing these disorders.

4.21. Comparison of Levels of Toxic and Essential Elements in Children with NDDs and NT Children

The concentrations of toxic and essential elements in children with NDDs and children without NDDs were different as shown in Table 4.21. The toxic (Al, Pb and As) and Mn mean levels in children with NDDs were significantly higher when compared to that of NT children ($p < 0.000$; $p < 0.000$; $p < 0.000$; and $p < 0.000$). The mean concentrations of essential elements, Mg, Ca, V, Zn, Se and Cu were significantly reduced respectively in children with NDDs compared to those in NT children ($p < 0.001$; $p < 0.000$; $p < 0.002$; $p < 0.000$; $p < 0.000$ and $p < 0.002$). In addition, the Zn/Cu ratio was significantly lower in children with NDDs ($p < 0.000$) relative to that of NT.

4.22. Comparison of Levels of Neurotransmitters and Oxidative Stress Markers in Children with NDDs And NT Children

The analyses of levels of neurotransmitters and oxidative stress markers in children with NDDs and controls were summarized in Table 4.22. The result showed that glutamine level in NDDs was substantially reduced ($p < 0.001$) when compared to the control, while glutamate/glutamine ratio increased significantly ($p < 0.001$) in children with NDDs than in NT children. The glutamate and GABA concentrations and GABA/glutamine ratio showed no significant differences between the two groups. Furthermore, the mean level of TAC was reduced significantly ($p<0.001$), while levels of malondialdyde ($p < 0.001$) and OSI ($p < 0.003$) were elevated significantly in children with NDDs when compared with NT children. (see Table 4.22).

Table 4.20: Comparison of Developmental Milestones Between Children with NDDs and NT Children Using Student T-Test

Variables	NDDs (N=50)	NT (N=25)	T-value	P-value
Stable neck (months)	5.84±5.13	3.00±0.96	2.729	**0.008***
Sitting (months)	8.74±6.99	4.82±1.22	2.774	**0.007***
Crawling (months)	9.98±6.17	6.16±1.46	3.034	**0.003***
Walking (months)	15.16±5.82	11.68±2.50	2.817	**0.007***
Talking (months)	26.81±15.96	13.04±3.86	4.201	**<0.001***

***Significant at p<0.05**

NDDs – Neuro-Developmental Disorders

NT – Neuro-Typical

Table 4.21: Comparison of Toxic and Essential Trace Metals Between Children with NDDs and NT Children Using Student T-Test

Toxic/trace elements	NDDs (N=50)	NT (N=25)	T-value	P-value
Pb (µg/dl)	10.28±5.02	5.43±2.04	4.634	**<0.001***
Al (µg/dl)	1.25±0.70	0.27±0.25	6.798	**<0.001***
As (µg/dl)	5.13±0.98	3.47±1.03	6.788	**<0.001***
V (µg/dl)	0.48±0.26	0.70±0.31	-3.184	**0.002***
Mg (mg/dl)	2.68±0.57	3.13±0.43	-3.546	**0.001***
Ca (mg/dl)	7.79±1.47	9.78±1.27	-5.757	**<0.001***
Zn (µg/dl)	228.04±86.32	438.5±185.5	-6.727	**<0.001***
Se (µg/dl)	34.23±9.86	59.03±5.28	-11.732	**<0.001***
Mn (µg/dl)	0.19±0.21	0.05±0.07	3.050	**0.003***
Cu (µg/dl)	4.16±0.91	4.88±0.94	-3.206	**0.002***
Zn/Cu	57.94±24.95	92.29±44.57	-4.286	**<0.001***

***Significant at p<0.05**

NDDs – Neuro-Developmental Disorders

NT – Neuro-Typical

Table 4.22: Comparison of Neurotransmitters and Oxidative Stress Markes Between Children with NDDs and NT Children Using Student T-Test

Neurotransmitters/ oxidative stress markers	NDDs (N=50)	NT (N=25)	T-value	P-value
Glutamine (µmol/l)	337.76±69.83	419.1±71.8	-4.714	**<0.001***
Glutamate (nmol/ml)	1.84±0.26	1.71±0.25	1.968	0.053
GABA (µmol/l)	1.93±0.41	1.77±0.25	1.839	0.070
Gte/Glu	0.006±0.001	0.004±0.001	4.325	**<0.001***
GABA/Gte	1.06±0.22	1.06±0.26	-0.021	0.983
Total Plasma Peroxidase	110.55±7.70	110.4±7.9	0.092	0.927
Total Antioxidant Capacity	245.03±59.06	303.8±33.1	-4.619	**<0.001***
Malondialdyde ($x10^{-5}$)	2.18±0.22	1.42±0.13	15.550	**<0.001***
Oxidative Stress Index	0.49±0.20	0.37±0.05	3.125	**0.003***

***Significant at p<0.05**

Gte/Glu – Glutamate/ Glutamine ratio

4.22.1. **Comparison of Biodata Variables Between ASD And NT Children**

4.23. Analysis of biodata variables in children with ASD and NT were summarized in Table 4.23. The results revealed there were no significant differences in all the variables (child's age, maternal age at the child's birth, paternal age at the child's birth, child's current weight) between ASD and CP children. However, the analysis revealed that ASD children had higher birth weight compared to NT children.

4.24. Comparison of Onset of Developmental Milestones Between ASD And NT Children

Developmental milestones analysis between ASD and NT children was summarized in Table 4.24. There were differences in onset of stable-neck, sitting, crawling and talking in ASD compared to NT ($p < 0.008$; $p < 0.006$; $p < 0.001$; $p < 0.001$).

4.25. Comparison of Levels of Toxic and Essential Elements in ASD And NT Children

Tables 4.25 summarizes comparison of toxic and essential metals between NT and ASD children. Toxic metals (Pb, Al and As) were increased significantly in ASD children when compared with NT ($p < 0.001$; $p < 0.001$; $p < 0.001$). Essential elements (Mg, Ca, V, Zn, Se and Cu) were significantly reduced ($p < 0.001$; $p < 0.001$; $p < 0.010$; $p < 0.001$; $p < 0.001$; $p < 0.049$) while Mn was elevated significantly ($p < 0.023$) in ASD children than NT children.

4.26. Comparison of Levels of Neurotransmitters and Oxidative Stress Makers in ASD and NT Children

According to the result in Table 4.26, glutamine and total antioxidant capacity were reduced significantly ($p < 0.030$; $p < 0.017$) while glutamate, GABA, glutamate/glutamine ratio, total plasma peroxide and Malondialdyde were significantly elevated ($p < 0.010$; $p < 0.001$; $p < 0.005$; $p < 0.010$; $p < 0.001$) in ASD compared with NT. There were no differences in GABA/Glutamate ratio and oxidative stress markers between the two groups.

Table 4.23: Comparison of Biodata Variables Between ASD and NT Groups (Post-hoc)

Variables	ASD (N=25)	NT (N=25)	T-value	P-value
Child's age (yrs.)	5.96±1.45	6.18±2.2.59	-0.370	0.713
Maternal's age at child's birth (yrs.)	26.68±2.69	27.96±2.70	-1.680	0.100
Paternal's age at child's birth (yrs.)	31.72±2.98	32.32±3.86	-0.615	0.541
Number of siblings	1.72±0.79	1.60±0.91	0.497	0.622
Child's weight (kg)	19.64±3.99	19.00±5.18	0.490	0.627
Child's birth weight (kg)	3.35±0.28	3.03±0.63	2.309	**0.025***

***Significant at p<0.05**

ASD: Autism Spectrum Disorder

CP: Cerebral Palsy

Table 4.24: Comparison of Onset of Developmental Milestones Between ASD and NT Groups (Post-hoc)

Variables	ASD (N=25)	NT (N=25)	T-value	P-value
Stable-neck	3.76±0.97	3.00±0.96	2.789	**0.008***
Sitting	5.68±0.85	4.82±1.22	2.897	**0.006***
Crawling	7.72±1.49	6.16±1.46	3.740	**<0.001***
Walking	12.76±2.99	11.68±2.50	1.387	0.172
Talking	27.67±6.60	13.04±3.86	9.148	**<0.001***

***p<0.05 is Significant**

Table 4.25: Comparison of Toxic and Essential Elements Between ASD and NT Children Using Post-hoc

Toxic/trace elements	ASD (N=25)	NT (N=25)	T-value	P-value
Pb (µg/dl)	9.49±4.04	5.43±2.04	4.483	**<0.001***
Al (µg/dl)	1.18±0.67	0.27±0.25	6.426	**<0.001***
As (µg/dl)	5.19±1.02	3.47±1.03	5.933	**<0.001***
V (µg/dl)	0.48±0.28	0.70±0.31	-2.693	**0.010***
Mg (mg/dl)	2.53±0.46	3.13±0.43	-4.830	**<0.001***
Ca (mg/dl)	7.91±1.38	9.78±1.27	-4.972	**<0.001***
Zn (µg/dl)	222.3±63.8	438.5±185.5	-5.511	**<0.001***
Se (µg/dl)	40.84±7.86	59.03±5.28	-9.601	**<0.001***
Mn (µg/dl)	0.17±0.24	0.05±0.07	2.353	**0.023***
Cu (µg/dl)	4.32±1.02	4.88±0.94	-2.020	**0.049***
Zn/Cu	55.31±22.04	92.29±44.57	-3.719	**0.001***

***p<0.05 is Significant**

Table 4.26: Comparison of Neurotransmitters and Oxidative Stress Makers Between ASD and NT Children (Post-hoc)

Neurotransmitters/ Oxidative stress markers	ASD (N=25)	NT (N=25)	T-value	P-value
Glutamine (µmol/l)	379.2±53.1	419.1±71.8	2.236	**0.030***
Glutamate (nmol/ml)	1.88±0.18	1.71±0.25	2.695	**0.010***
GABA (µmol/l)	2.07±0.34	1.77±0.25	3.612	**0.001***
Gte/Glu	0.005±0.001	0.004±0.001	2.932	**0.005***
GABA/Gte	1.11±0.18	1.06±0.26	0.762	0.450
Total Plasma Peroxidase	105.9±2.3	110.4±7.9	-2.679	**0.010***
Total Antioxidant Capacity	280.2±34.4	303.8±33.1	-2.468	**0.017***
Malondialdyde ($x10^{-5}$)	2.27±0.23	1.42±0.13	16.127	**<0.001***
Oxidative Stress Index	0.38±0.05	0.37±0.05	1.083	0.284

***Significant at p<0.05**

4.27. Comparison of Biodata Variables Between CP and NT Children

Table 4.27 shows the analysis of biodata variables in CP and NT children. The results revealed no notable variations in the variables between CP and NT groups.

4.28. Comparison of Onset of Developmental Milestones Between CP and NT Children

Onset of developmental milestones variables between CP and NT children were summarized in Table 4.28. The results found significant differences inonset of stable-neck ($p < 0.001$), sitting ($p < 0.001$), crawling ($p < 0.001$), walking ($p < 0.001$) and talking ($p < 0.033$)between CP and NT.

4.29 Concentrations of Toxic and Essential Elements in CP And NT Children

Table 4.29 shows the summary of analysis of toxic and trace elements in CP and NT groups. The results revealed that Mg, Ca, V, Zn, Se, Cu and Zn/Cu ratio were significantly reduced ($p < 0.049$; $p < 0.001$; $p < 0.011$; $p < 0.001$; $p < 0.001$; $p < 0.011$ and $p < 0.004$) while Pb, Al, Mn and As concentrations were significantly elevated ($p < 0.001$; $p < 0.001$; $p < 0.001$ and $p < 0.001$) in CP group compared to NT children.

4.30 Comparison of Neurotransmitters and Oxidative Stress Markers in CP and NT Children

The comparative analysis of data on neurotransmitters and oxidative stress markers between children with CP and NT was summarized in Table 4.30. The results showed a significant ($p < 0.001$) increase in glutamate concentration and a significant ($p < 0.001$) decrease in glutamate/glutamine ratio in children with CP compared to NT. However, the analysis of results of markers of oxidative stress showed that total plasma peroxide, malondialdyde and oxidative stress index levels were significantly elevated ($p < 0.045$; $p < 0.001$; $p < 0.001$) while total antioxidant capacity was significantly reduced ($p<0.001$) in children with CP compared with NT children.

Table 4.27: Comparison of Biodata Variables Between CP and NT Groups (Post-hoc)

Variables	CP (N=25)	NT (N=25)	T-value	P-value
Child's age (yrs.)	5.12±3.03	6.18±2.59	-1.329	0.190
Maternal age at child's birth (yrs.)	27.76±4.88	27.96±2.70	-0.179	0.858
Paternal age at child's birth (yrs.)	33.28±6.52	32.32±3.86	0.633	0.530
Number of child's siblings	1.64±1.19	1.60±0.91	0.134	0.894
Child's weight (kg)	16.94±8.07	19.00±5.18	-1.072	0.289
Child's birth weight (kg)	2.90±0.73	3.03±0.63	-0.693	0.492

***Significant at p < 0.05**

Table 4.28: Comparison of Developmental Milestones Between CP and NT Groups (Post-hoc)

Variables	CP (N=25)	NT (N=25)	T-value	P-value
Stable-neck	8.72±6.97	3.00±0.96	4.071	**<0.001***
Sitting	13.00±9.31	4.82±1.22	4.361	**<0.001***
Crawling	13.50±8.73	6.16±1.46	4.145	**<0.001***
Walking	19.77±7.18	11.68±2.50	5.123	**<0.001***
Talking	25.11±27.02	13.04±3.86	2.231	**0.033***

***p < 0.05 is Significant**

Table 4.29: Comparison of Toxic and Essential Elements Between CP and NT Children (Post-hoc)

Trace elements	CP (N=25)	NT (N=25)	T-value	P-value
Pb (µg/dl)	11.07±5.81	5.43±2.04	4.581	**<0.001***
Al (µg/dl)	1.32±0.74	0.27±0.25	6.739	**<0.001***
As (µg/dl)	5.06±0.96	3.47±1.03	5.689	**<0.001***
V (µg/dl)	0.49±0.25	0.70±0.31	-2.646	**0.011***
Mg (mg/dl)	2.82±0.64	3.13±0.43	-2.022	**0.049***
Ca (mg/dl)	7.68±1.56	9.78±1.27	-5.192	**<0.001***
Zn (µg/dl)	233.8±105.3	438.5±185.5	-4.800	**<0.001***
Se (µg/dl)	27.62±6.76	59.03±5.28	-18.314	**<0.001***
Mn (µg/dl)	0.21±0.20	0.05±0.07	3.712	**0.001***
Cu (µg/dl)	4.00±0.77	4.88±0.94	-3.630	**0.001***
Zn/Cu	60.57±27.77	92.29±44.57	-3.021	**0.004***

***p < 0.05 is Significant**

Table 4.30: Comparison of Levels of Neurotransmitters and Oxidative Stress Markers between CP and NT Children (Post-hoc)

Neurotransmitters/ Oxidative stress markers	CP (N=25)	NT (N=25)	T-value	P-value
Glutamine (µmol/l)	296.3±59.6	419.1±71.8	-6.581	**<0.001***
Glutamate (nmol/ml)	1.80±0.33	1.71±0.25	1.031	0.308
GABA (µmol/l)	1.79±0.43	1.77±0.25	0.241	0.810
Gte/Glu	0.006±0.002	0.004±0.001	5.122	**<0.001***
GABA/Gte	1.01±0.24	1.06±0.26	-0.711	0.480
Total Plasma Peroxidase	115.1±8.5	110.4±7.9	2.060	**0.045***
Total Antioxidant Capacity	209.8±57.9	303.8±33.1	-7.050	**<0.001***
Malondialdyde ($x10^{-5}$)	2.08±0.17	1.42±0.13	15.373	**<0.001***
Oxidative Stress Index	0.60±0.23	0.37±0.05	5.042	**<0.001***

***p <0.05 is Significant**

4.31 Comparison of Biodata Variables Between ASD and CP Children

Table 4.31 summarizes and compare the biodata variables of ASD and CP children. There were no significant variations in all the biodata variables except child's birth weight which showed significant increase ($p < 0.006$) in ASD than CP group.

4.32 Comparison of the Onset of Developmental Milestones Between ASD and CP Children

Onset of developmental milestones variables between children with ASD and CP were summarized in Table 4.32. The results showed there were significant differences in onset of stable-neck ($p < 0.001$), sitting ($p < 0.001$), crawling ($p < 0.002$) and walking ($p < 0.001$) between children with ASD and CP.

4.33 Comparison of Toxic and Essential Elements Between ASD and CP Children

Table 4.33 shows the comparative analysis of levels of essential and toxic elements between the case I (ASD) and case II (CP). The results showed no major variations in all the elements analyzed except plasma Se, which was reduced in children with CP compared to ASD children ($p < 0.001$).

4.34 Comparison of Levels of Neurotransmitters and Oxidative Stress Markers Between ASD and CP Children

Summary of neurotransmitters and oxidative stress markers concentrations were shown in Table 4.34. The results showed a significant reduction in the levels of glutamine ($p < 0.000$), GABA ($p < 0.014$), totalantioxidant capacity ($p < 0.001$) and Malondialdyde ($p < 0.001$) in CP than in ASD children. However, TPP, OSI and glutamate/glutamine ratio were significantly elevated ($p<0.001$; $p<0.001$; $p<0.001$) in CP compared to ASD.

Table 4.31: Comparison of Biodata Variables Between ASD and CP Children (Post-hoc)

Variables	ASD (N=25)	CP (N=25)	T-value	P-value
Child's Age (yrs.)	5.96±1.45	5.12±3.03	1.250	0.217
Maternal's age at child's birth (yrs.)	26.68±2.69	27.76±4.88	-0.970	0.337
Paternal's age at child's Birth (yrs.)	31.72±2.98	33.28±6.52	-1.088	0.282
Number of child's siblings	1.72±0.79	1.64±1.19	0.281	0.780
Child's weight (kg)	19.64±3.99	16.94±8.07	1.498	0.141
Child's birth weight (kg)	3.35±0.28[b]	2.90±0.73	2.907	**0.006***

***Significant at p < 0.05**

Table 4.32: Comparison of Developmental Milestones Between ASD and CP Groups (Post-hoc)

Variables	ASD (N=25)	CP (N=25)	T-value	P-value
Stable-neck	3.76±0.97	8.72±6.97	-3.529	**0.001***
Sitting	5.68±0.85	13.00±9.31	-3.926	**<0.001***
Crawling	7.72±1.49	13.50±8.73	-3.261	**0.002***
Walking	12.76±2.99	19.77±7.18	-4.262	**<0.001***
Talking	27.67±6.60	25.11±27.02	0.386	0.703

***p < 0.05 is Significant**

Table 4.33: Comparison of Toxic and Essential Elements Between ASD and CP children (Post-hoc)

Toxic/trace elements	ASD (N=25)	CP (N=25)	T-value	P-value
Pb (µg/dl)	9.49±4.04	11.07±5.81	-1.117	0.270
Al (µg/dl)	1.18±0.67	1.32±0.74	-0.676	0.502
As (µg/dl)	5.19±1.02	5.06±0.96	0.441	0.661
V (µg/dl)	0.48±0.28	0.49±0.25	-0.153	0.879
Mg (mg/dl)	2.53±0.46	2.82±0.64	-1.879	0.066
Ca (mg/dl)	7.91±1.38	7.68±1.56	0.541	0.591
Zn (µg/dl)	222.3±63.8	233.8±105.3	-0.466	0.643
Se (µg/dl)	**40.84±7.86**	**27.62±6.76**	**6.379**	**<0.000***
Mn (µg/dl)	0.17±0.24	0.21±0.20	-0.603	0.549
Cu (µg/dl)	4.32±1.02	4.00±0.77	1.268	0.211
Zn/Cu	55.31±22.04	60.57±27.77	-0.741	0.462

***p < 0.05 is Significant**

Table 4.34: Comparison of Neurotransmitters Levels and Oxidative Stress Makers Between ASD and CP Children (Post-hoc)

Neurotransmitters /Oxidative Stress markers	ASD (N=25)	CP (N=25)	T-value	P-value
Glutamine (µmol/l)	379.2±53.1	296.3±59.6	5.189	**0.001***
Glutamate (nmol/ml)	1.88±0.18	1.80±0.33	1.078	0.287
GABA (µmol/l)	2.07±0.34	1.79±0.43	2.553	**0.014***
Gte/Glu	0.005±0.001	0.006±0.002	-3.390	**0.001***
GABA/Gte	1.11±0.18	1.01±0.24	1.621	0.112
Total Plasma Peroxide	105.9±2.3	115.1±8.5	-5.229	**<0.001***
Total Antioxidant Capacity	280.2±34.4	209.8±57.9	5.228	**<0.001***
Malondialdyde ($x10^{-5}$)	2.27±0.23	2.08±0.17	3.484	**0.001***
Oxidative Stress Index	0.38±0.05	0.60±0.23	-4.684	**<0.001***

***Significant at p<0.05**

4.35 Correlations Between Toxic and Essential Elements as well as Neurotransmitters and Markers of Oxidative Stress in ASD Children

Tables 4.1.35 summarises the correlations of toxic and essential elements with neurotransmitters and markers of oxidative stress in ASD. Significant positive correlations were found between Mg and Zn/cu ratio (0.589, 0.002), Mn and GABA (0.532, 0.006), Zn and Zn/Cu ratio (0.839, 0.001), Cu and glutamine (0.455, 0.022), while negative and significant correlations were observed between Mg and GABA (-0.47, 0.016) as well as Cu and Zn/Cu ratio (-0.785, 0.001).

4.36 Correlations Between Toxic and Essential Elements as well as Neurotransmitters and Markers of Oxidative Stress in CP Children

Table 4.36 displays summary of correlations analysis of toxic and essential elements with neurotransmitters and oxidative stress markers in CP children. Glutamate (0.511, 0.009), glutamate/glutamine ratio (0.536, 0.006) and Zn/Cu ratio (0.417, 0.038) were positively and significantly correlated with Cu respectively. There were positive and significant correlations between Zn and Zn/cu ratio (0.906, 0.001) as well as between Al and TPP (0.433, 0.031).

4.37 Correlations Between Toxic and Essential Elements as well as Neurotransmitters and Markers of Oxidative Stress in NT Children

Tables 4.37 summarises the correlations of toxic and trace elements with neurotransmitters and oxidative stress markers in NT. According to the findings of this study, there were direct relationships between Mg and glutamine (0.401, 0.049), Zn and Zn/Cu ratio (0.907, 0.001) as well as Se and GABA/Glutamate ratio (0.499, 0.011), while negative correlation existed between Mg and OSI (-0.438, 0.029), Se and Glutamate (-0.549, 0.004) and Se and Glutamate/Glutamine ratio (-0.638, 0.001).

Table 4.35: Correlation of Toxic and Trace Elements with Neurotransmitters and Oxidative Stress Markers in ASD Children

		Glu	Gte	GABA	TPP	TAC	MDA	OSI	Gte/Gl	GABA/ Gt	Zn/Cu
Mg	R	-0.019	-0.247	-0.477	-0.182	0.058	0.006	-0.092	-0.167	-0.329	0.589
	P	0.927	0.235	**0.016***	0.384	0.784	0.979	0.663	0.424	0.108	**0.002***
Ca	R	0.151	-0.039	-0.060	-0.088	-0.338	-0.018	0.306	-0.125	-0.063	0.134
	P	0.470	0.855	0.777	0.675	0.098	0.932	0.137	0.550	0.765	0.522
Pb	R	0.013	0.060	-0.249	-0.109	-0.105	-0.036	0.039	0.018	-0.279	0.048
	P	0.950	0.777	0.230	0.602	0.619	0.866	0.853	0.933	0.177	0.821
Al	R	-0.167	0.095	-0.116	-0.188	0.236	-0.045	-0.242	0.158	-0.163	0.194
	P	0424	0.652	0.582	0.369	0.256	0.830	0.243	0.452	0.435	0.352
V	R	-0.209	0.105	-0.093	-0.122	-0.152	0.212	0.136	0.249	-0.165	0.221
	P	0.315	0.617	0.657	0.560	0.468	0.308	0.518	0.229	0.431	0.288
Zn	R	0.070	-0.009	0.188	-0.128	0.147	0.260	-0.172	-0.026	0.180	0.839
	P	0.741	0.966	0.367	0.542	0.483	0.209	0.411	0.902	0.389	**0.001***
Se	R	-0.065	-0.201	-0.020	-0.096	0.084	0.041	-0.037	-0.101	0.100	-0.037
	P	0 .758	0.335	0.923	0.650	0.689	0.846	0.861	0.632	0.635	0.862
Mn	R	0.013	0.281	0.532	-0.177	0.350	0.181	0.356	0.202	0.341	0.115
	P	0.949	0.173	**0.006***	0.397	0.086	0.386	0.081	0.332	0.096	0.585
Cu	R	0.455	0.218	0.060	0.384	0.202	0.095	-0.151	-0.220	-0.067	-0.785
	P	**0.022***	0.294	0.777	0.058	0.334	0.653	0.472	0.290	0.749	**0.001***
As	R	-0.062	-0.210	-0.350	-0.062	-0.083	0.107	0.106	-0.126	-0.220	-0.251
	P	0.767	0.313	0.086	0.768	0.693	0.611	0.615	0.547	0.291	0.227

***Significant at p<0.05**

Table 4.36: Correlation of Essential and Toxic Elements with Neurotransmitters and Oxidative Stress Markers in CP

		Glu	Gte	GABA	TPP	TAC	MDA	OSI	Gte/Gl	GABA/ Gte	Zn/Cu
Mg	R	-0.220	-0.222	-0.142	-0.219	-0.162	0.070	-0.078	0.081	0.041	0.001
	P	0.291	0.287	0.498	0.293	0.438	0.739	0.712	0.701	0.847	0.995
Ca	R	-0.110	-0.146	0.166	-0.246	-0.265	-0.166	0.315	0.018	0.226	0.283
	P	0.600	0.486	0.427	0.236	0.201	0.429	0.126	0.932	0.278	0.170
Pb	R	-0.342	-0.179	-0.156	-0.203	0.229	-0.151	-0.258	0.159	0.002	-0.376
	P	0.094	0.392	0.456	0.331	0.271	0.470	0.214	0.447	0.992	0.064
Al	R	0.265	0.328	0.131	0.433	0.051	-0.252	-0.032	-0.042	-0.104	-0.325
	P	0.200	0.110	0.533	**0.031***	0.808	0.225	0.877	0.842	0.619	0.113
V	R	0.146	0.062	0.188	0.165	0.241	-0.248	-0.086	-0.061	0.111	-0.200
	P	0.487	0.767	0.368	0.430	0.246	0.233	0.683	0.773	0.598	0.337
Zn	R	0.071	-0.087	0.079	-0.024	-0.102	0.075	0.105	-0.127	0.154	0.906
	P	0.737	0.680	0.707	0.908	0.628	0.723	0.619	0.547	0.463	**<0.001***
Se	R	-0.184	0.213	-0.141	-0.133	-0.308	-0.129	0.242	0.278	-0.313	-0.021
	P	0.378	0.307	0.501	0.526	0.135	0.537	0.244	0.178	0.127	0.920
Mn	R	0.037	-0.245	-0.048	-0.157	-0.005	-0.088	0.003	-0.131	0.188	0.107
	P	0.859	0.237	0.819	0.453	0.982	0.675	0.988	0.534	0.368	0.612
Cu	R	-0.239	0.511	0.300	0.491	0.185	-0.100	-0.002	0.536	-0.112	0.417
	P	0.250	**0.009***	0.145	**0.013***	0.375	0.635	0.991	**0.006***	0.593	**0.038***
As	R	-0.178	-0.271	0.085	0.058	0.045	-0.054	-0.022	-0.005	0.284	0.016
	P	0.396	0.191	0.685	0.784	0.830	0.799	0.916	0.982	0.169	0.939

***Significant at p<0.05**

Table 4.37: Correlation of Toxic and Essential Elements with Neurotransmitters and Oxidative Stress Markers in NT Children

		Glu	Gte	GABA	TPP	TAC	MDA	OSI	Gte/Gl	GABA/ Gte	Zn/Cu
Mg	R	0.401	0.226	-0.054	-0.337	0.291	0.284	-0.438	-0.153	-0.179	0.378
	P	**0.047***	0.278	0.796	0.099	0.158	0.169	**0.029***	0.467	0.391	0.063
Ca	R	-0.130	0.246	-0.148	-0.220	-0.085	-0.318	-0.022	0.195	-0.277	-0.125
	P	0.535	0.235	0.480	0.290	0.686	0.121	0.917	0.350	0.180	0.551
Pb	R	-0.283	0.313	-0.152	-0.195	-0.165	0.303	-0.004	0.379	-0.280	0.238
	P	0.151	0.128	0.470	0.350	0.431	0.140	0.986	0.062	0.175	0.252
Al	R	-0.296	-0.020	-0.088	-0.015	0.084	-0.266	-0.083	0.221	-0.026	-0.166
	P	0.151	0.926	0.677	0.943	0.689	0.198	0.692	0.289	0.901	0.428
V	R	0.372	-0.151	0.027	-0.254	0.310	0.044	-0.372	-0.339	0.124	0.196
	P	0.067	0.470	0.898	0.221	0.132	0.835	0.067	0.097	0.555	0.348
Zn	R	0.046	0.197	-0.313	0.055	0.158	0.192	-0.106	0.091	-0.336	0.907
	P	0.827	0.345	0.128	0.793	0.452	0.357	0.613	0.664	0.101	**<0.001***
Se	R	0.447	-0.549	0.174	-0.239	0.208	-0.127	-0.270	-0.638	0.499	-0.011
	P	**0.025***	**0.004***	0.405	0.251	0.319	0.544	0.191	**0.00***	**0.011***	0.959
Mn	R	-0.068	-0.341	-0.026	0.216	0.324	-0.146	-0.131	-0.180	0.262	-0.194
	P	0.746	0.095	0.901	0.300	0.114	0.486	0.532	0.389	0.206	0.354
Cu	R	-0.312	0.017	0.208	0.222	-0.077	-0.133	0.170	0.246	0.090	-0.302
	P	0.128	0.935	0.318	0.287	0.715	0.527	0.417	0.236	0.668	0.142
As	R	-0.210	-0.175	0.072	-0.074	-0.086	-0.290	0.039	0.037	0.160	-0.354
	P	0.314	0.403	0.732	0.725	0.683	0.159	0.854	0.862	0.444	0.082

***Significant at p<0.05**

4.38 Test of Hypotheses

Hypothesis 1

Statement: There will be no major variations in environmental exposed factors between occupationally exposed and unexposed pregnant women.

Alpha level: 0.05

Test statistics: chi-square

X^2 = 0.252; 1.170; 0.001; 0.786; 2.085; 0.521; 1.174

P = 0.615; 0.557; 0.969; 0.375; 0.352; 0.471; 0.759

Decision: since P in all the variables > 0.05, the hypothesis was NOT REJECTED. It may be concluded that there were no major variations in environmentally exposed factors between occupationally exposed and unexposed pregnant women.

Hypothesis 2

Statement: There will be no significant difference in socio-economic status between occupationally exposed and unexposed pregnant women.

Alpha level: 0.05

Test statistics: chi-square

X^2 = 87.652; 97.555; 14.440; 19.515

P = 0.001; 0.001; 0.002; 0.000

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were major variations in Socio-Economic Status between occupationally exposed and unexposed pregnant women.

Hypothesis 3

Statement: There will be no major differences in toxic (Pb, Cd) metals concentrations between exposed and unexposed pregnant women

Alpha level: 0.05

Test statistics: paired t-test

T = 1.000; -0.519;

P = 0.425; 0.604

Decision: since P in the variables > 0.05, the hypothesis was NOT REJECTED. There were no major variations in toxic (Pb, Cd) metals concentrations in exposed and

unexposed pregnant women.

Hypothesis 4

Statement: There will be no significant difference in essential (Cu, Zn, Ca, Mg and Se) elements levels in occupationally exposed and unexposed pregnant women.
Alpha level: 0.05

Test statistics: paired t-test

T = -0.780; -0.978; 0.034; 1.088; -0.528

P = 0.437; 0.338; 0.973; 0.279; 0.597

Decision: since P in the variables > 0.05, the hypothesis was NOT REJECTED. There were no notable variations in essential (Cu, Zn, Ca, Mg and Se) elements concentrations between occupationally exposed and unexposed pregnant women.

Hypothesis 5

Statement: There will be no significant differences in cord blood essential (Cu, Zn, Ca, andSe) elements concentrations between exposed and unexposed groups.
Alpha level: 0.05

Test statistics: paired t-test

T = -0.807; -1.028; -0.291; -1.235

P = 0.420; 0 . 3 0 6 ; 0.772; 0.217

Decision: since P in the variables > 0.05, the hypothesis was NOT REJECTED. It was concluded that there were no significant differences in essential (Cu, Zn, Ca, and Se) elements concentrations between occupationally exposed and unexposed pregnant women.

Hypothesis 6

Statement: There will be no significant correlation in toxic metals levels between maternal and cord blood
Alpha level: 0.05

Test statistics: Pearson Correlation

Decision: since the toxic metals was not detectable in more than 90% of the cord blood,

the hypothesis could not be tested. Thus, it was DISCARDED.

Hypothesis 7

Statement: There will be no significant correlation in levels of essential elements in maternal and cord blood.

Alpha level: 0.05

Test statistics: Pearson correlation,

r = 0.266; 0.450; -0.349

P = 0.045; 0.005; 0.022

Decision: since P < 0.05 in the variables (maternal Zn Vs Cord Mg; maternal Se Vs Cord Cu; maternal Ca Vs Cord Se), the hypothesis was REJECTED. It was concluded that there were significant correlations in levels of essential elements between maternal and cord blood.

Hypothesis 8

Statement: There will be no significant difference in socio-economic factors among ASD, CP and NT.

Alpha level: 0.05

Test statistics: Pearson Chi-square

X^2 = 23.985; 16.592; 21.031; 24.284

P = 0.001; 0.002; 0.002; 0.000

Decision: since P < 0.05 in the variables, the hypothesis was REJECTED. It was concluded that there were notable variations in socio-economic factors among ASD, CP and NT groups.

Hypothesis 9

Statement: There will be no significant difference in medical and health issues among ASD, CP and NT

Alpha level: 0.05

Test statistics: Pearson Chi-square

X^2 = 13.043; 50.543; 57.555; 37.500; 8.451; 15.082

P = 0.001; 0.000; 0.000; 0.000; 0.015; 0.020

Decision: since P < 0.05 in the test variables, the hypothesis was REJECTED. It was

concluded that there were major variations in medical and health issues within ASD, CP and NT groups.

Hypothesis 10

Statement: There will be no significant differences in environmental and household dust exposure factors among ASD, CP and NT.

Alpha level: 0.05

Test statistics: Pearson Chi-square

X^2 = 0.000; 2.440; 1.316

P = 1.000; 0.655; 0.518

Decision: since P > 0.05 in test variables, the hypothesis was NOT REJECTED. It was concluded that there were no major variations in environmental and household dust exposure factors among ASD, CP and NT.

Hypothesis 11

Statement: There will be no significant difference in biodata variables between NDDs and NT. Alpha level: 0.05

Test statistics: paired t-test

T = -1.063; -0.845; 0.156; 0.336; -0.477; 0.627

P = 0.291; 0.401; 0.877; 0.738; 0.635; 0.533

Decision: since P > 0.05 in test variables, the hypothesis was NOT REJECTED. It was concluded that there were no significant differences in biodata variables between NDDs and NT.

Hypothesis 12

Statement: There will be no significant difference in toxic metals (Pb, Al, As) concentrations between children with NDDs and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = 4.634; 6.798; 6.788

P = 0.000; 0.000; 0.000

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there was significant difference in toxic metals (Pb, Al, As) concentrations between children with NDDs and NT children.

Hypothesis 13

Statement: There will be no significant difference in micronutrients (Mg, Ca, V, Zn,Se, Mn, Cu) levels between NDDs and NT children.

Alpha level: 0.05

Test statistics: paired t-test

t = -3.546; -5.757; -3.184; -6.727; -11.732; 3.050; -3.206

P = 0.001; 0.000; 0.002; 0.000; 0.000; 0.003; 0.002

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were major variations in micronutrients (Mg, Ca, V, Zn, Se, Mn, Cu) levels between NDDs and NT children.

Hypothesis 14

Statement: There will be no significant difference in neurotransmitters (glutamate, GABA) levels between NDDs and NT children.

Alpha level: 0.05

Test statistics: paired t-test = 1.968; 1.839;

P = 0.053; 0.070;

Decision: since P in the variables > 0.05, the hypothesis was NOT REJECTED. It was concluded that there were no significant differences in neurotransmitters (glutamate, GABA) levels between NDDs and NT children.

Hypothesis 15

Statement: There will be no significant difference in oxidative stress markers (TAC, MDA, OSI) concentrations between NDDs and NT children.

Alpha level: 0.05

Test statistics: paired t-test= -4.619; 15.550; 3.125

P = 0.000; 0.000; 0.003

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in oxidative stress markers (TAC, MDA, OSI) concentrations between NDDs and NT children.

Hypothesis 16

Statement: There will be no notable variations in biodata variables between ASD and NT. Alpha level: 0.05

Test statistics: paired t-test

t = -1.370; -1.680; 0.615; 0.497; 0.490

P = 0.713; 0.100; 0.541; 0.622; 0.627

Decision: since P > 0.05 in test variables, the hypothesis was NOT REJECTED. It was concluded that there were no notable variations in biodata variables in ASD and NT.

Hypothesis 17

Statement: There will be no significant differences in toxic metals (Pb, Al, As) concentrations between ASD and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = 4.483; 6.426; 5.933

P = 0.000; 0.000; 0.000

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in toxic metals (Pb, Al, As) concentrations between ASD and NT children.

Hypothesis 18

Statement: There will be no significant differences in micronutrients (Mg, Ca, V, Zn, Se, Mn, Cu) levels between ASD and NT groups.

Alpha level: 0.05

Test statistics: paired t-test

T = -4.830; -4.972; -2.693; -5.511; -9.601; 2.353; -2.020

P = 0.000; 0.000; 0.010; 0.000; 0.000; 0.023; 0.049

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in micronutrients (Mg, Ca, V, Zn, Se, Mn, Cu) concentrations in ASD and NT groups.

Hypothesis 19

Statement: There will be no significant differences in neurotransmitters (glutamate, GABA) levels between children with ASD and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = 2.695; 3.612

P = 0.010; 0.001

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was

concluded that there were significant differences in neurotransmitters (glutamate, GABA) levels in children with ASD and NT children.

Hypothesis 20

Statement: There will be no significant difference in oxidative stress markers (TAC, MDA, TPP) concentrations between ASD and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = -2.468; 16.127; -2.679

P = 0.017; 0.000; 0.010

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in oxidative stress markers (TAC, MDA, TPP) concentrations in children with ASD and NT.

Hypothesis 21

Statement: There will be no notable variations in biodata variables between children with CP and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = -1.329; -0.179; 0.633; 0.134; -1.072

P = 0.190; 0.858; 0.530; 0.894; 0.289

Decision: since P > 0.05 in test variables, the hypothesis was NOT REJECTED. It was concluded that there were no notable variations in biodata variables in children with CP and NT children.

Hypothesis 22

Statement: There will be no significant difference in toxic metals (Pb, Al, As) concentrations between children with CP and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = 4.581; 6.739; 5.689

P = 0.000; 0.000; 0.000

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in toxic metals (Pb, Al, As) concentrations between children with CP and NT children.

Hypothesis 23

Statement: There will be no significant difference in micronutrients (Mg, Ca, V, Zn,Se, Mn, Cu) levels between children with CP and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = -2.022; -5.192; -2.646; -4.800; -18.314; 3.712; -3.630

P = 0.049; 0.000; 0.011; 0.000; 0.000; 0.001; 0.001

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in micronutrients (Mg, Ca, V, Zn, Se, Mn, Cu) levels between children with CP and NT children.

Hypothesis 24

Statement: There will be no significant differences in neurotransmitters (glutamate, GABA) levels between children with CP and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = 1.031; 0.241

P = 0.308; 0.810

Decision: since P in the variables > 0.05, the hypothesis was NOT REJECTED. It was concluded that there were no significant differences in neurotransmitters (glutamate, GABA) concentrations in children with CP and NT children.

Hypothesis 25

Statement: There will be no significant differences in oxidative stress markers (TAC, MDA, OSI, TPP) concentrations between children with CP and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = -7.050; 15.373; 5.042; 2.060

P = 0.000; 0.000; 0.000; 0.045

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in oxidative stress markers (TAC, MDA, OSI, TPP) concentrations between CP and NT children.

Hypothesis 26

Statement: There will be no major variations in biodata variables between children with

ASD and CP.

Alpha level: 0.05

Test statistics: paired t-test

T = 1.250; -0.970; -1.088; 0.281; 1.498

P = 0.217; 0.337; 0.282; 0.780; 0.141

Decision: since P > 0.05 in test variables, the hypothesis was NOT REJECTED. It was concluded that there were no notable variations in biodata variables between children with ASD and CP children.

Hypothesis 27

Statement: There will be no significant differences in toxic metals (Pb, Al, As) concentrations in children with ASD and CP.

Alpha level: 0.05

Test statistics: paired t-test

T = -1.117; -0.676; 0.441

P = 0.270; 0.502; 0.661

Decision: since P in the variables > 0.05, the hypothesis was NOT REJECTED. It was concluded that there were significant differences in toxic metals (Pb, Al, As) concentrations between children with ASD and CP children.

Hypothesis 28

Statement: There will be no significant differences in trace elements (Mg, Ca, V, Zn, Mn, Cu) concentrations between children with CP and NT children.

Alpha level: 0.05

Test statistics: paired t-test

T = -1.879; 0.541; -0.153; -0.466; -0.603; 1.268

P = 0.066; 0.591; 0.879; 0.643; 0.549; 0.661

Decision: since P in the variables > 0.05, the hypothesis was NOT REJECTED. It was concluded that there were significant differences in trace elements (Mg, Ca, V, Zn, Mn, Cu) concentration in ASD and CP children.

Hypothesis 29

Statement: There will be no significant differences in neurotransmitters (glutamine, GABA) levels in ASD and CP children.

Alpha level: 0.05

Test statistics: paired t-test

T = 5.189; 2.553

P = 0.000; 0.014

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in neurotransmitters (glutamine, GABA) levels in ASD and CP children.

Hypothesis 30

Statement: There will be no significant difference in oxidative stress markers (TAC, MDA, OSI TPP) concentrations in ASD and CP children.

Alpha level: 0.05

Test statistics: paired t-test

T = 5.228; 3.484; -4.684; -5.229

P = 0.000; 0.001; 0.000; 0.000

Decision: since P in the variables < 0.05, the hypothesis was REJECTED. It was concluded that there were significant differences in oxidative stress markers (TAC, MDA, OSI, TPP) concentrations between ASD and CP children.

CHAPTER FIVE

DISCUSSION

5.1. DISCUSION

In this work, pregnant mothers environmentally exposed to toxicants like Pb, Cd and essential elements like Ca, Mg, Zn, Se and V were evaluated; possible placental transfer of these metals through the umbilical cord into the fetal circulation *in-utero* was also investigated. In consonance with established biochemical and physiological roles of these elements in metabolic processes, efforts were made to identify and control possible confounding factors using both demographic and statistical tools.

The results of anthropometric data of occupationally exposed and unexposed pregnant women implied that the groups were comparable and the difference implied that the exposed pregnant women married at a younger age and had their first child earlier than the unexposed. This may also be due to lower educational status in the CP group. The groups were exposed to similar environmental toxicants and this may therefore not be confounding results on exposure to occupational toxicants recorded in this study.

The result of nutritional and dietary supplementations implied that both the exposed and unexposed pregnant women were consuming similar diet and nutritional supplements and this may not confound the results of exposure to toxicants as recorded in the study.

Recruitment of participants to this project was done with due consideration for the credibility and appropriateness of the reports of this study because anthropogenic and anthropometric variables are recognized sources of environmental toxicants and pollutants (Alloway, 2012). Many pollutants and toxicants have been linked to occupational exposure of the above variables, including cadmium through smoking and seafoods, lead obtained from leaded paints, insecticides as well as environmental dust (Jaga & Dharmani, 2003; Cecchi *et al*., 2012; Ye *et al*., 2017). Thus, participants recruited for this work were compared taking into consideration the kind of housing and proximity to a tarred road they live in, pesticides usage, smoking (active or passive) as well as exposure to different environmental pollutants derived from dust. This was to ensure that the results to be obtained were not confounded. Other variables, such as the

use of nutritional supplements and consumption of sea food, particularly in pregnancy, were also evaluated and found to be comparable in all of the subjects, according to the results of the provided structured questionnaire. It was necessary to limit possible exposure to harmful elements such as cadmium, that may be derived from marine foods, as well as other critical components included in most nutritional supplements freely taken by pregnant women, particularly in this region of the globe.

Furthermore, consumption of various nutritional supplements indiscriminately may cause alteration in some essential elements concentrations resulting in oxidant/antioxidant imbalance within the system. This study observed similarities in nutritional supplements consumption in the participants recruited. Also, mothers' and their spouses' educational grades were evaluated, this was to determine the social group and educational standard of the participants recruited. These were also found to be similar in the participants. In conclusion, the participants' anthropometric and anthropogenic variables [cases (ASD and CP) and controls] in this study were similar. This showed that the choice of participants for this project was appropriate to ensure the veracity and conclusion made from the biochemical results obtained in the study. This became imperative especially to allow for extrapolation of the from *in-utero* exposure to developing baby traits.

5.1.1. Essential Trace Elements in Environmentally Exposed Pregnant Women

Pregnancy which is often referred to as “Gestation” is the period during which offspring(s) develops inside a woman’s womb after sexual intercourse although assistedreproductive technology procedures now exist to ensure implantation without sexual intercourse. Normally and usually, gestational period lasts for about 40 weeks duringwhich the fertilized egg(s) develops as an embryo to become a foetus until birth (Abman, *2011*). It is usually divided into 3 trimesters each lasting for approximately 3months under normal circumstances. Many developmental processes in the child’ssystem occur during this foetal stage, however; abnormality in any of these developmental stages either genetically or environmentally imposed may not manifest until the early years of the child after birth. Since the baby derived her nutrients and other body needs from the mother through the umbilical cord, post-birth developments in the baby are usually influenced by whatever might have been transferred to the baby from the mother during the uterine life (Punshon *et al.,* 2016; Zaw & Taneepanichskul, 2019). Hence, various health problems of the baby

manifesting in the early years may be traced to various materials to which the baby is exposed during intra-uterine life; ASD,CP and even other neurodevelopmental and neurodegenerative diseases are no exceptions. As stated above, one of the main hypotheses of this work (1.5.1.1.) was that "Cord blood toxic (Pb and Cd) and essential (Se, Zn, Cu, Mg and Ca) elements concentrations reflected maternal levels based on placental transfer mechanism". Since cord blood reflects foetal blood circulation, it may be concluded from results of this work that elements were transferred from mothers' circulations into the baby's system during uterine life. According to a detailed examination of the degree of transfer that impacted all elements under review, Zn was especially substantially transmitted from maternal into the unborn child's circulation. The amount of Zn in the infant's cord blood was considerably higher than in the mother from whom it was received, indicating that it accumulated more in the newborn. This discovery was in line with previous studies, which indicated that neonates had a greater Zn level in cord blood than mothers (Irwinda *et al*., 2019). Cu levels were likewise increased in exposed babies' cord blood than in unexposed newborns, in contrast to maternal blood Cu levels, which were increased in unexposed maternal circulation than in the exposed maternal blood. Although previous studies have shown similar results, the significance of this report is that cord blood Cu should have the same pattern because the infant acquired the nutrients from the maternal circulation while still in the womb (Irwinda *et al*., 2019). Zn and Cu shared an inverse correlation, specifically when one surpasses the other (Halsted, *et al*., 1968). As a result, considering the metabolic importance of these two metals, a reasonable explanation for this occurrence is required, especially since it may explain some challenges connected to neurogenesis and its other disorders.

The elements recognized to play key roles in neurogenesis' metabolic processes are Zn, Se, and Mg as their functions in axon growth and antioxidation are well documented (Adamo & Oteiza, 2010). Although, Cu is thought to have a role in a variety of neurodevelopmental processes and is required for the efficient functioning of various enzymes in the brain. Copper toxicity and deficiency can affect brain development and function. The association between the increased cord Cu level and both Zn and Se, which work together to balance oxidant/antioxidant ratio in the brain may then be investigated (de Lucia *et al*., 2020). Zn is understood to partner with Cu in the Cu/Zn enzyme dismutase. This enzyme scavenges toxicants in the form of ROS, which can disrupt the oxidant/antioxidant equilibrium in the brain, along with glutathione. The

scavenging activity is anchored by metallothionein for which the two metals maintain a balanced ratio in their concentration (which normally is almost 1:1) to perform this function (de Lucia *et al*., 2020). The association between the increased cord Cu level and both Zn and Se, which work together to balance the oxidant/antioxidant ratio in the brain may then be viewed along the above theory.

However, the high level of Zn in the cord blood found in this work may be due to increased mobilization of Zn from the maternal blood for the synthesis of Zn-based antioxidant enzymes and molecules required to combat an environmentally-induced oxidative stress. This is based on the fact that a considerable quantity of Zn was transported from the maternal to the foetal circulation, as evidenced by the babies' cord blood level (exposed and unexposed). Another plausible inference from this could be that the Zn ions essential for neurogenesis may not have been present at the cellular level to accomplish the required co-enzymatic activity. This might be due to the non-development of various Zn-related enzymatic pathways with age, resulting in a lack of Zn at the cellular level to complement Se, both of which are the primary antioxidant pools in neurons. Conversely, it may be hypothesized that Zn-related enzymes are not really participating in the required metabolic activities of a growing fetus *in-utero*, either due to their immaturity of Zn-related enzymes or because they are completely unavailable at that moment. Hence, it may be suggested that the antioxidant mechanism in newborns' brains focuses on Se and Mg levels rather than Mg, Zn and Se levels at the point. Even a higher Zn level in the cord blood of unexposed newborns than in exposed babies confirms the fact that Zn is indeed required in oxidative balance process in the developing brain of newborns and required in about 360 enzymes, some of which are very important during infant stage.

It has been well established that Zn and Cu blood levels should be equal for appropriate metallothionein activity, especially for the optimization of its scavenging ability against excess ROS caused by metal toxicants. Although this antioxidative activity in neurons has been shown to be mostly dependent on Zn and Se, its ineffectiveness has been linked to an increase in blood Cu or a reduction in blood Zn levels (Pokusa & Tranková, 2017). If the metallothionein system was functioning, a rise in Zn level as measured in cord blood of exposed newborns in this study should results in a commensurate decrease in Cu level in blood of those newborns.

In furtherance to the above hypothesis, the lack of this complementary impact of Zn and Se may overwhelm the latter's capability as evidenced by its much lower cord blood concentration in exposed in comparison to that od the unexposed maternal and cord blood. Mg is known to protect brain from chemical toxicity, whereas a deficit is thought to make heavy metal poisoning severe. The synthesis of glutathione, an essential antioxidant mechanism in the body, requires an optimal Mg level and this has been linked to the aetiology of learning difficulties in children (Drybanska-Kalita,1995). This loss in anti-oxidative power caused by reduced Se and low/non-consequential Zn concentration may have overwhelmed the ability of Mg in exposed newborns, causing a decrease in exposed cord blood Mg as compared to unexposed.

The interaction of Se and Mg as the principal antioxidant element in the uterine stage in exposed newborns may also be inferred since Mg concentrations in the exposed and non-exposed mothers' circulation were almost identical. The significant reduction in Mg levels reported in the exposed newborns might be causing a depletion of the body's antioxidant pool, particularly glutathione levels. Thus, the findings showed that Se is the major antioxidant metal in the neuron at the uterine level particularly in occupationally and environmentally exposed pregnant women and its deficiency may lead to abnormal neurodevelopment, eventually causing neurodevelopmental disorders such as ASD and CP in infancy.

5.1.2. Gender Differences in ASD and CP

ASD has been reported to be more prevalent in boys than in girls (Loomes *et al.,* 2017). In this study, this was also confirmed as male to female ratio found in children with ASD was 5:1. This may be due to the fact that females have a factor on XX chromosome that is protective of developing ASD. Males having higher prevalent can be due to X and Y genes on their sex chromosomes, these genes may be up or down regulated due to cellular mechanisms that affect gene expression. It has been reported that females are more intelligent than males, and furthermore female ASD patients that do not have any physical or cerebral impairments (Schaafsma & Pfaff, 2014). The prevalence of ASD in boys in this study agreed with previous studies that reported high ASD prevalence in boys than in girls (Baron-Cohen *et al.,* 2011; Loomes *et al.,* 2017; Zeidan *et al.,* 2021). In the same pattern, CP was also found to be more prevalent in boys than in girls. The male to female ratio found in this study was 3:1. The higher prevalence in males than in

females may also be due to sex hormone estrogen (E2) in females. Estrogen possesses antioxidant capabilities that result from its capacity to bind to estrogen receptors and to activate intracellular signalling pathways that up-regulate the production of antioxidant enzymes (Borrás et al., 2010). Estrogen's ability to directly scavenge free radicals in the body cells may explain its antioxidant impact. (Ruiz-Larrea et al., 1997). Different cell types experience the protective effects of estrogen. Different cellular CNS models, including glial and neuronal cells, show that E2 raises glutathione levels. (Schmidt et al., 2002). The estrogen in females may protect them more from the attack of oxidative stress.

5.1.3. Essential (Ca, Mg, Zn, Cu, Mn, Se, V) and toxic (Pb, As, Al) elements levels in clinically diagnosed neurodevelopmental (ASD and CP) disorders.

The second hypothesis of this work focusses on the effect of essential and toxic elements on the function of neurotransmitters and balance of oxidative stress markers on the aetiopathogenesis of neurodevelopmental disorders (ASD and CP) in children. It should be observed that in the broad sense, the hypothesis is combining the effect of trace elements and toxic metals on neurotransmitters and oxidative stress markers activities respectively. Hence, discussion here will focus on the effect of the above parameters on NDDs generally and specifically on ASD and CP disorders.

Analysis of the data revealed that in NDDs there was general upregulations in toxic elements (Pb, Al, As) levels while that of essential elements (Ca, Mg, Zn, Se, Cu, V) were downregulated with the exception of Mn that was significantly higher in blood circulation of children with NDDs. This may be due to the active metabolic processes (including neurogenesis) that is ongoing in the early life of a developing child. The increased level of Mn may also be due to increase mobilization of Mn-based antioxidants enzymes and molecules in response to high level of toxicants particularly from the toxic metals inducing oxidative stress. Pb, As and Al are known toxicants that may interfere with many metabolic processes including neurogenesis (Tran & Miyake, 2017).

Exposure to these toxicants is commonly through ingestion or inhalation which on a continuous basis may have deleterious effect especially in a developing economy where there is little or no control on exposure to environmental toxicants coming with quantity of food. This may result in accumulation of these environmental toxicants, the

deleterious effect of which may be pathological in vulnerable group like developing children. The role of most of these essential elements as co-enzymes and facilitators in many metabolic processes has been severally documented (Kirschning et al., 2012). As previously stated, Zn, Se, Mg and Cu very important in the metabolic processes of neurogenesis as antioxidants and function in many enzymatic processes involved in brain neurodevelopmental (Adamo & Oteiza, 2010; Cecilia *et al.*, 2012). Therefore, the observed down regulation of essential elements in children with NDDs in this work may not be unconnected with their rapid depletion due to active metabolic activities (including neurogenesis) in these children. This may underscore the epigenetic theory linking the effect of exposure to environmental/occupational toxicants to genetic modification as the basis of NDDs.

Mn is one of the essential metals necessary for proper *foetal* development (Wood, 2009). Mn has also been confirmed to be a component of Mn catalase and Mn superoxide dismutase, enzymes that help to reduce oxidative stress by detoxifying superoxide-derived free radicals (Li & Zhou, 2011). In spite of this function, excess amount of this metal in the body may actually be due to increase mobilization of the Mn-based antioxidants enzymes and molecules in response increased level of toxicants particularly toxic metals found in the participants in this study which can inducing oxidative stress. Although, it has also been reported that high level on Mn could be toxic causing neurological impairment (O'Neal & Zheng, 2015). Hence, the observed elevated Mn level in children with NDDs in this study may be another contributory factor to the progression of NDDs in the children. Among the major sources of exposure to Mn as advised by the WHO were drinking water and environmental dust (WHO, 2012); however, the results of anthropometric data obtained from the participants showed no significant variation in the quality of drinking water by NDDs and NT children. On the other hand, the result of anthropometric data showed a significant variation in the exposure of the NDDs and NT children to environmental dust. It may therefore be inferred that elevated Mn level was as a result of continuous exposure to Mn through dust facilitating the exacerbation of neurodevelopmental disorder in the children.

5.1.4. Essential (Ca, Mg, Zn, Cu, Mn, Se, V) and toxic (Pb, As, Al) Elements levels in clinically diagnosed ASD, CP and NT children.

Autism Spectrum Disorders are type of neuro-developmental disorders that impairs

persons communication and social interaction. The individual with ASD also displays restricted range of habits or repetitive behavior. As stated earlier, (CP) is a physical, permanent life-long disability that affects movement and posture. Epigenetic factors have now been connected to the development of all these neurological disorders (Blake *et al.*, 2013; Siniscalco *et al*., 2013). Due to its unclear pathophysiology and the ambiguous findings of genetic mapping in the diagnosis, the recent rise in these disorders has caused concern around the world. As a consequence, there seems to be an increasing interest in learning more about the pathophysiology of environmental toxicants as well as trace elements in the aetiopathogenesis of ASD and CP. Determination of essential (Zn, Mg, Ca, Se, Cu, Mn and V) and toxic (Cd, Al and As) elements was done to evaluate possible influence of these metals on the various biomarkers that can be used in the management of these disorders. Discussions of results of this work will look at the possible link between ASD and CP in comparison with NT since the two disorders are not only neurodevelopmental disorders but also share some clinical symptoms in common. Essentially, the possible anti-oxidative and neuroprotective roles of these metals' interaction were under consideration.

Every cell in the body requires magnesium as an important element for overall health. It is associated with axon production and stabilization and is needed for the maximum brain function and muscle cells (Yamanaka *et al.,* 2019). Signal carriage, that ensures signal transmission through one neuron to another, is among the most well-known roles of axons in neural transmission (Long & Romani, 2014). Magnesium has also been linked to the regulation of calcium entry into nerve cells (Carolyn Dean, 1995). In this work, Mg was reduced almost similarly in both ASD and CP children. The lower Mg concentration found in this study may be linked to abnormal axon growth and stabilization. This maybe an impediment in the exchange of signals, resulting in the typical repetitive behaviour shown by children with ASD. The clinical symptom of motor impairment characteristic of CP may also be as a result of reduced Mg since this metal is also known to be associated with muscle cell and axon development and stabilization. The physiological basis of this is imperative since for nerve signals to be properly interpreted and executed at the organ level, the impulse must be appropriately transmitted. This is the major function of the axon, an abnormality of which may have a deleterious effect on neurodevelopment manifesting as characteristic repetitive behaviour in children with ASD and impaired movement characteristic of CP. Long and

Romani (2014), reported that neuromuscular and neuropsychiatric disturbances are usually manifestations of magnesium deficiency. Although, most of the previous works on Mg in ASD and CP were done on hair and nail, similar works on blood Mg level reported a significant lower levels of Mg in children with ASD and CP (Omotosho *et al.*, 2018). Therefore, the observed low plasma magnesium level in this study may significantly be a contributory factor in the aetiopathogenesis of ASD

Calcium (Ca) is a macro element that is essential for neurodevelopment and synaptic plasticity. Calcium in its various forms is essential for many metabolic functions of the body. The total calcium fraction is associated with its skeletal function while ionized form is associated with various metabolic activities like muscle contractions, enzymes activities and nerve impulses. It is involved in normal neuromuscular function and have an important function in blood coagulation. Calcium is reported to be responsible for electrical signal transmission through nerves (Lohmann, 2009).

Reduced Ca levels were reported in ASD and CP children in this study. The hypocalcaemia could be caused by lower magnesium levels, as previously reported, which could result in irregular signal transmission along nerves in developing neurons during the prenatal and postnatal periods. It may also be stated that the function of Ca as a membrane-enzyme modulating element (Ca ATPase) may be compromised by the observed hypocalcaemia which on a continuous basis (as may be inferred in children in this study) may gradually disturb entrance of other essential nutrients at the cellular level including the neural cell. This phenomenon may be exacerbated by the reduced appetite- a physiological aberration especially associated with ASD. Therefore, since Ca is essentially obtained from diet, its reduction will not only affect Ca level but may also precipitate the down-regulation of other essential nutrients that Ca-modulated membrane-enzyme facilitates their entrance into the cells. The deleterious effect of this phenomenon may not be unconnected with the abnormal neurodevelopment and the attendant clinical symptoms associated with ASD and CP. Since, movement and posture disability are prominent features of CP, reduction in Ca, an important element associated with the skeletal frame of the body, reduction in Ca level may be a major contributory factor to these clinical symptoms of CP. As a result, persistent or sporadic hypocalcemia in humans can impair neurite outgrowth and synaptogenesis, predisposing to neurological changes that present as neuropsychiatric disorders. Although, the mechanism is not known, calcium dysregulation is increasingly being

implicated as a cause of ASD and CP (Napolioni, 2011; Zeidán-Chuliá, 2013). Where both genetic and environmental factors are considered, calcium dysregulation is implicated in many studies as one of the most common biological factors in neurodevelopmental pathogenesis (Zeidán-Chuliá, 2013; Long and Romani, 2014; Omotosho *et al.,* 2018). Therefore, the observed low plasma calcium concentration in this study may be a crucial contributory factor and biomarker in the aetiopathogenesis of ASD and CP.

One of the most important micro-elements studied in this work is copper. Ceruloplasmin is a copper-binding antioxidant protein that is present in the body, as well as the brain. It transports copper (Vassiliev *et al.,* 2005). Copper has two functions: it stimulates neurological activity and it aids the body's antioxidant defense system. It combines with Zn to form metallothionein, which is the major scavenger of toxicants in the nervous system, as part of its anti-oxidative function. Due to its simple changes from Cu^{+} to Cu^{++}, it also plays a crucial role in redox reactions. Due to this biochemical disposition of Cu (its possible conversion from Cu^{+} to Cu^{++}), the metal may function as both an oxidant and as an antioxidant. Hence, copper deficiency or toxicity may be damaging to the nervous system ultimately resulting in neurological diseases (Madsen & Gitlin, 2007; Gaetke *et al.,* 2014). In this work, the observed reduction in plasma copper level may be a factor accentuating one of the characteristic developmental abnormalities associated with ASD and CP. This essentially may be as a result of a deficiency of metallothionein which as earlier stated is needed for active ROS scavenging in neurogenesis. Thus, the reduced Cu may compromise synthesis of this enzyme leading to an imbalance in oxidant/antioxidant ratio and the attendant deleterious effect on neurogenesis. Copper levels in the cell may also influence protein synthesis by promoting or inhibiting the transcription of particular genes since copper is known to regulates genes expression through moderation of intracellular oxidative stress (Mattie *et al*., 2008). Since children in this study were at their active developmental stages involving active protein synthesis, any abnormality in gene regulation as may be induced by a deficiency in Cu level may also precipitate abnormal gene formation resulting in abnormal protein synthesis.

Previous works have reported disturbance in copper metabolism and altered Cu in ASD and CP (Russo & DeVito, 2011; Bjorklund, 2013; El-Baz *et al*., 2018). Many metal-analysis studies done to explore the disorders also reported a relationship in copper

metabolism and autism spectrum disorder (ASD) (Russo & DeVito, 2011; Bjorklund, 2013; El-Baz *et al.*,2018). Although the findings of this research contradicted those of other studies that found higher copper levels in ASD patients when compared to levels in controls (Russo*et al.*, 2012; El-Meshada *et al.*, 2017; Saldanha Tschinkel *et al.*, 2018), however, result of this work was similar to those of Kalra *et al*. (2015) and Craciun, *et al*. (2016) who observed a reduced Cu level in ASD and CP against the control respectively. Malnutrition which is a known predisposing factor that reduces Cu level (at least in this environment) was not observed in any of the children recruited for the study, this may also be inferred from similarity in both anthropometric and anthrogenic factors recorded for the children. Hence, Cu deficiency may be involved in the aetiopathogenesis of both ASD and CP, according to the findings.

Zinc is a trace element that can be found in the tissues and fluids of the body, it is important in various physiological and metabolic processes where its functions can be catalytic, structural or regulatory (Roohani *et al.*, 2013). Zinc is required for the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids, as well as the metabolism of other micronutrients. It is found in over 300 enzymes. Its role in polynucleotide transcription and, by extension, genetic expression is also important. Essentially, Zn is obtained from diet; hence, a deficiency of this element in the body either as a result of inadequate intake or increased requirement may produce abnormality. In this study, low Zn levels found in children with ASD and CP may be primarily due to inadequate intake and increased requirements necessitated by increased metabolic activities in the participants occasioned by growth. In most cases, a lack of absorbable zinc in the diet causes zinc deficit, which is widespread in many parts of the world (WHO, 2011; Veenemans *et al.*, 2011; Nair *et al.*, 2017). Reduced dietary intake or poor absorption rate according to studies, may exacerbate physiological conditions associated with elevated zinc requirements, such as in actively growing children (Veenemans *et al.*, 2011). Neurological changes have been linked to severe Zn deficiency as far back as 1972 by Halsted *et al*. Also, neuropsychological changes like emotional instability, irritability and depression including abnormal cognitive performance have all been linked with Zn deficiency in various studies. (Hambidge 2000; Russo & deVito 2011). One of the prominent functions of Zn is as a powerful antioxidant in neurogenesis through the formation of metallothionein which scavenges metal toxicants.

The low Zn level observed in this work may compromise this function, leading to an

imbalance in oxidant/antioxidant level in the children and its consequent abnormal neurodevelopment. Although most studies on Zn level in ASD and CP reported significant Zn variation in hair and nail of the children (Lakshmi & Geetha, 2011), this study would be one of very few in this environment focusing on blood micronutrients level in children with NNDs. The observed hypozincaemia may therefore be an important bio indicator of ASD and CP in this environment.

Selenium is a trace element required for the biosynthesis of several selenoproteins, like glutathione peroxidases and thioredoxin reductases, which function as antioxidants by catalyzing the breakdown of hydrogen peroxide, organic hydrogen peroxides, to form reduced protein disulfide bonds (Holmgren & Lu, 2010; Roman *et al.*, 2014). Due to its essential role, it is maintained in the brain even when dietary supply is low. In this study, children with ASD and CP both had reduced selenium levels, however, Se concentration in this study was very low in CP children even in comparison to children with ASD. This highly significant variations in Se concentration obtained in the two groups of children may be linked to the degree of neurological impairment associated with each of them. Typically, CP manifests with age long motor and cognitive impairments which may be related to the highly reduced antioxidant pool that reduced Se level precipitates. It may also not be unconnected with the earlier submission that Se is the main antioxidant protecting neurogenesis *in-utero*; hence, abnormality at this stage may be the genesis of the onset of permanent brain damage that eventually manifests as irreparable deficit in both motor and cognitive impairments, the main clinical feature of CP.

The attendant deficit in antioxidant pool could have played a vital role in the pathogenesis of ASD due to the fact that glutathione, a powerful antioxidant found in the brain's neuro-endocrine tissues may also become depleted affecting neural transmission and behaviour. Glutathione is a major redox buffer in the neuroendocrine tissue's transsulfuration pathway (James, *et al.*, 2004). Glutathione and metallothionine are the major sources of antioxidants that scavenge metal toxicants in neurogenesis. Therefore, a reduction in Se as seen in this study may precipitate deficiency of selenoproteins with its attendant reduction in glutathione activities. As a result, it is possible that a low Se level disrupts this mechanism, which could explain many of the neuro-behavioural changes connected with ASD and CP. The reduction in Se level found in this study was in agreement with previous studies of Jory and Woody, 2008; Pyria and Geetha, 2010;

Blaurock-Busch *et al.,* 2012, that reported low Se levels in ASD and CP children hair and red blood cells when compared to controls. Above notwithstanding, a divergent opinion on interpretation of reduced Se especially in some parts of the world including Africa has been suggested. In a review article by Schomburg, (2016), the author submitted that there is a general low selenium level in African children. Hence, the lower selenium concentration found in this study may need to be investigated further before it may be used as a biomarker in children with NDDs.

Although Vanadium was reported as essential trace mineral since 1971 and its main exposure source to the populace was said to be food (Fortoul *et. al.,* 2014), Lower types of life have vanadium-activated enzymes, only recently confirmed that vanadium serves a similar role in higher animals. A few studies on the element revealed that it is a mineral element that is required by animals for normal growth and development, as well as having an insulin-like action *in vivo* (Srivastava *et al.,* 2005; Treviño *et al.,* 2019). Vanadium has been said to be involved in the regulation of some vital enzymes like Na+/K+-exchanging ATPase, and protein kinases in biochemical studies (Ścibior *et al.,* 2020). The concentration of the vital element was found to be reduced in ASD and CP children in this research. The concentration was significantly reduced when compare to that of NT children.

As a result, lower concentration recorded in ASD and CP may be associated with underdevelopment of the brain's motor and cognitive functions in these two neurodevelopmental disorders. Also, since the element has been reported as a cofactor in the pumping activity of Na^+/K^+ATPase, reduced level of this element may also affect the known activity of this enzyme which is largely responsible for maintaining the inner milieu of the cells that will create a conducive environment for other metabolic processes (Ścibior *et al.,* 2020). A disturbance in the ionic configuration and balance in an organ like the brain, especially during the formative stages, may proof so fatal as to precipitate biochemical derangement that may manifest in cognitive and behavioural abnormality in later life as seen in both children with ASD and CP.

Manganese (Mn) is one of the essential microelements investigated in this study. Among its other functions, it plays a role in neurogenesis by converting glutamate to glutamine, a process catalyzed by glutamine synthetase, which requires Mn (Hertz, 2013). Deficiency and excess Mn have been associated with various metabolic

dysfunction. However, excess amount of Mn has been associated with the production of an imbalance in the antioxidant level leading to accumulation of ROS in the nervous tissue (Martinez-Finley *et al.,* 2013). The observed increase in Mn level in this research in children with ASD and CPmay also have linked to the oxidant/antioxidant imbalance which become accentuated with accumulation of glutamate. As it has been established, glutamate is an excitatory neurotransmitter, hence, its accumulation may be the basis of excessive neural excitation which may be manifesting in the known clinical symptoms of ASD and CP. Previous findings in both animal and human experiments have all linked excess Mn level with many of the known clinical signs and symptoms of ASD and CP (Arora *et al*., 2017; WHO, 2012).

Aluminium concentration was found to be higher in the two neurodevelopmental cases studied in this work (ASD and CP); however, it was higher in CP children than in ASD children. Since Al has been reported to interfere with Ca metabolism, it may be inferred that the disruptive effect of Al on Ca metabolism may be more pronounced in CP subjects in comparison to ASD cases. This may be explained by the observed pronounced memory impairment and neurological disorders which may be the outcome of disruption in mineralization and bone growth associated with children with CP (Gonzalez-Weller *et al.,* 2010; Exley, 2013; Exley and Vickers, 2014; Martinez *et al.*, 2017; Alexey *et al.,* 2019; Exley and Clarkson, 2020). The more pronounced hypocalcaemia reported in children with CP in this study gave credence to the deduction. On the other hand, the increased Al concentration found in ASD in this study may be a contributory factor to the neurological disorder especially cognitive damage which is the major clinical signs of ASD. This abnormality may also be associated with the lower Ca concentration seen in ASD. Since Ca is an essential element in neural impulse transmission, a process that is defective in children with ASD.

Several studies done on human hair have linked ASD and CP to aluminium exposure (Yasuda *et al.,* 2013; Rahbar *et al.,*2016; Skalny *et al.*, 2017; Alexey *et al.*, 2019) and blood and urine are used to a limited extent (Sulaiman *et al*., 2020). These studies agree with the findings in the present work. Mold *et al*. (2018) reported intracellular aluminium associated with non-neuronal cells in autism brain tissue. A veritable source of exposure to Al in this environment may be the popular use of Al cooking utensils, a practice that has gained a lot of propensity in this environment. Since Al as a microelement can easily traverse the blood brain barrier of baby either *in-utero* or early

neonatal period.

Lead (Pb) is an ubiquitous environmental pollutant which has been reported to have no known safe level in humans particularly in children. It causes serious contamination of the environment and affect health (WHO, 2015). Its deleterious effects on virtually every organ in the body such as the nervous system have been reported. The toxic effects of this metal are accentuated by its ability to displace some essential metals in many metabolic processes. Since Pb crosses the placenta causing adverse health with the brain being most vulnerable (Naeher *et al.,* 2004). Also, low levels of lead can cause behavioural issues, cognitive delays, and reduced IQ in infants and young children (Bas *et al.,* 2015). The toxic metal lead (Pb^{2+}) was reported to trigger cognitive and behavioural impairments, resulting in various neurological symptoms (Lanphear, 2005; Sanders *et al.,* 2009). Also, the increased vulnerability of children to air-borne metallic lead than adults may be due totheir higher respiration and metabolic rates (WHO 2011), Thus, making the toxic effect of the metal amplified in children, where it interferes with normal brain development.

In this study, lead levels in children with neurodevelopmental disorders (ASD and CP) were significantly elevated compared to the level in NT children. Lead toxicity is known to be prevented by the presence of adequate amounts of essential elements like calcium, magnesium, zinc and copper. However, since these essential elements are largely derived from diet, their deficiency which has been reported to be physiological in children with NNDs may be precipitated by the poor eating habits may have exacerbated Pb toxicity in children with NNDs.

The hypocalcaemia and hypozincaemia recorded in the study (Omotosho *et al,* 2018), may be secondary to exposure of these children to toxic lead level, thereby abnormal neurogenesis due to the displacement of the essential metals in various metabolic processes. The continued exposure to the toxicant, Pb, may be associated with the progression of the abnormality at the vulnerable age of the children ultimatelyresulting in the various abnormal clinical signs of ASD and CP. In previous hair studies, ASD group had higher lead levels than controls (Gorini *et al*., 2014; Adam *et al.,* 2009), blood lead levels in ASD and CP children were higher than those found in NT children in this report, as well. This was also in agreement with studies of Clark *et al.* (2010) and George *et al. (*2010) that reported increased blood levels in ASD. Airborne and settled dusts

constitute good surrogates of air pollution and reflect the potential risk of general public exposure to organic and inorganic pollutants. (Mohmand *et al.*, 2015). Lead's potential to substitute for other bivalent cations such as Ca^{2+}, Mg^{2+},and Fe^{2+}, which are all involved in body is physiological and metabolic processes as fundamental to its ionic mechanism of action (Flora *et al*., 2012).

Protein folding, maturation, apoptosis, ionic transportation, enzyme regulation, and neurotransmitter released have all been shown to be significantly affected (Garza *et al*., 2006) by lead. Lead, after replacing calcium ions, becomes capable of crossing the blood-brain barrier (BBB) at a significant rate, which leads primarily to neurological deficits. Also, when lead substitutes for calcium, it inappropriately triggers processes reliant on calmodulin thereby interfering with intracellular calcium release from the mitochondria (Sanders et al., 2009; Virgolini & Aschner, 2021). Acetylcholine, dopamine, and amino acid neurotransmitters are all inhibited by this andare activated by Ca^{2+}, but also increases release from the basal ganglia (Lester *et al*., 2010). Although the mechanism is unclear, lead has been shown to affect presynaptic Ca^{2+} channels involved in transmitter release and by activating protein kinase C (PKC) (Bouton *et al*., 2001). Also, the elevated blood level of lead found in this study could be linked to brain's redox function disturbance. Complex processes underlie lead-induced neurotoxicity. The main factors contributing to Pb neurotoxicity include oxidative stress, membrane biophysics changes, dysregulation of cell signaling, and impairment of neurotransmission (Sanders *et al.*, 2009). Lead toxicity can result from oxidative stress and directly or indirectly generated lipid peroxidation (Khan *et al.*, 2008). This is because high blood lead levels have been linked to a range of synaptic functions and structures (Mason et al., 2014; Lisa *et al.*, 2014; Brokes *et al.*, 2004). The production of reactive oxygen species (ROS), such as hydroperoxides, singlet oxygen, and hydrogen peroxide, as well as the direct depletion of antioxidant reserves, are two distinct, though related, routes by which lead toxicity causes free radical damage. Glutathione, a cysteine-based chemical formed in the internal space of the cell, is one of the effects of lead exposure. Glutathione's sulfhydryl complex binds to hazardous metals with a strong affinity for sulfhydryl groups. Thus, the glutathione molecule can be effectively inactivated by lead, rendering it useless as an antioxidant (Sanders *et al.*, 2009). Lead binds to enzymes with active sulfhydryl groups, leaving them inactive and adding to the disturbance of oxidative equilibrium. Both animal and human lead exposure investigations revealed decreased levels of glutathione reductase and ALAD,

two particular sulfhydryl-containing enzymes that are inhibited by lead (Sanders et al., 2009). It also disrupts GABAergic, dopaminergic, and cholinergic functional systems by interfering with neurotransmitter release (Lisa *et al.*, 2014).

The possibility of "reduced tolerance" by some individuals to lead toxicity has also been suggested. Some authours have proposed that persons with ASD were not the only ones who have been exposed to lead, and that increased levels of toxic heavy metals in tissue in children with ASD may result from a greater propensity to absorb toxins, leading to a change in biochemical processes (Adams *et al.,* 2009; Filon *et al.,* 2020; Baj *et al.*, 2021). As a consequence, children with neurodevelopmental conditions including autism spectrum disorder and cerebral palsy may possibly haveissues with the chemical system that helps in detoxifying metals in order to relieve a number of autistic symptoms (Priya & Geetha, 2011). Although the research is ambiguous, evidence suggests that autistic children in particular have an elevated build-up of toxins, which may be from body's failure to eliminates toxins rather than simply from prolonged exposure. Such a mechanism may cause toxic heavy metals accumulation, as well as the chemical toxins that come with them, resulting in free radical activity elevation in the body (Naumann & colleagues, 2005). There have been few studies along this line, particularly in humans. Thus, supplementation with elements that can chelate toxic metals like Pb may likely reduce the toxic metal levels and improves the severity of neurodevelopmental disorders.

Increased lead levels in NNDs children in this study may be due to environmental factors rather than occupational hazards. According to the analysis of the data collected, 16 percent of ASD children and 68 percent children with CP live on an untarred street, respectively. Lead poisoning may occur from a variety of causes, including leaded gasoline and paint, lead-contaminated dust and soil as well as water obtained from leaded pipes, industrial contamination, and occupational exposure according to Tong and others (2007). Therefore, environmental exposure to Pb as seen from the questionnaire used to obtain demographic data on these children may be a major source of lead contamination.

5.1.5. Neurotransmitters in ASD and CP

In the course of this work, chemical neurotransmitters were determined towards establishing their possible link with essential/toxic metals and oxidative markers in the

pathogenesis and progression of these two neurodevelopmental disorders. Hence, in pursuant of the second hypothesis of this work which states “Toxic and essential trace elements altered neurotransmitters and oxidative stress markers, thus, associated and connected to the aetiopathogenesis of neurodevelopmental disorders (ASD and CP) in children” (**1.5.1.2.)**, a reappraisal of the anatomic and physiologic structure of the brain and spinal cord in relation to neurotransmitters and nerve impulse transmission becomes imperative. The brain serves as the body's main control module, orchestrating everything from physical activity to hormone secretion, memory development, and emotional sensations (NINDS, 2020; Newman, 2017). Some parts of the brain are specifically designed to carry out these functions. Many higher functions, on the other hand entail different areas cooperating in networks. Effective control and moderation of these functions is carried out with the aid of nerves running from the CNS to the various organs and targets in the body. These nerves carry impulses generated from thebrain transmitting them from one nerve to the other via synaptic junctions which effect transfer of information using neurotransmitters. As previously stated, the CNS has been severally classified and consists of many supporting cells that are essential for effective neural activity; among these is the astrocytes which play very significant function in the maintenance of excitatory and inhibitory balance needed for normal nerve impulse transmission (Kim *et al.,* 2019; Liu *et al.,* 2021). Hence, abnormality in these cells greatly affect generation and synthesis of neurotransmitters with the attendant effect on message transfer from one region of the body to the other.

Although many neurotransmitters have been investigated in the search for the diagnosis and management of neurodevelopmental diseases, in this study, GABA, Glutamate and Glutamine were estimated in clinically established ASD and CP individuals compared to NT. GABA is an established inhibitory neurotransmitter while glutamateis also an established excitatory neurotransmitter (Bjørklund, 2013; Naaijen *et al.,* 2017). These two neurotransmitters maintain nerve impulse by ensuring excitation and inhibition appropriately to ensure normalcy in neural activity through the glutamate- glutamine-GABA (Gte/Glu-GABA) cycle. It has been established that in neurons, the enzyme glutaminase produces glutamate from glutamine (Coughlan *et al.,* 2015; Horder *et al.,* 2018). The glutamate formed is then released and picked back by astrocytes, where glutamine synthetase converts it to glutamine, which is then transferred and reused in neuron (Al-Otaish *et al.,* 2018). GABA is formed via the decarboxylation of glutamate

as a mechanism to prevent the accumulation of glutamate in brain cells.

The Enzyme- Glutamic acid decarboxylase (GAD), an important mitochondrial enzyme related to glutamate excitotoxicity (Coughlan *et al.*, 2015). The glutamate/GABA process has GABA as the rate-limiting enzyme thus making this glutamate–glutamine-GABA cycle a vital factor of the glutamatergic neurotransmission system. This cycle essentially maintains active conversion of glutamate to glutamine, it may therefore be postulated that over excitation of the neurons which may be directly due to over activity of glutamate or underactivity due to excessive inhibition by GABA may be involved in the aetiogenesis of neurodevelopmental disorders and the basis of the clinical symptoms of repetitive behaviour, cognitive dysfunction and hyperactivity common in children with NDDs.

With the above picture of the anatomical, physiological and biochemical integration in the CNS, the excitatory effect of the glutamatergic neurotransmitter (glutamate) may be seen to be more predominant than the inhibitory effect of the GABAergic neurotransmitter (GABA). The findings of this study demonstrate this, indicating a reduction in amount of amino acid glutamine which incidentally is the precursor or foundation of the glutamate-glutamine-GABA cycle responsible for maintaining a balanced excitatory/inhibitory nerve impulse in the CNS. This observed reduction between children with ASD and those with CP and those NT may be due to a reduction or gross deficiency of the mitochondrial enzymes, glutamine synthetase that catalyse the conversion of glutamate to glutamine. The sustained decarboxylation of the limited glutamine in these children by the enzyme glutamine decarboxylase in the astrocytes may be the basis of the increased GABA levels (a neuro-inhibitory transmitter) obtained in this study in ASD and CP when compared to NT. Theaccumulation of GABA which was more prevalent in ASD children than in CP children may have accentuated accumulation of glutamate as indicated by the Gte/Glu ratio resulting in hyper-excitation and its attendant increased hyperactivity at the target organs and sites of the neural impulse. This inference may be the basis of the clinical symptoms of hyper and repetitive behaviour and other sensory abnormalities which are more pronounced in children with ASD than in those with CP.

Results of elevated levels of plasma Mn in a prevailing environment of reduced plasma Ca, Se and Mg further strengthen the above deductions because these will further create

antioxidant/oxidant imbalance which may further disrupt enzymatic activities during nerve impulse generation and transmission. The deductions from this work were similar to studies of Al-Otaish *et al. (*2018) and Marotta *et al. (*2020) -who found elevated glutamate and GABA in NDDs.

5.1.6. OXIDATIVE STRESS IN ASD AND CP

Excessive free radical production or a dysfunctional antioxidant system causes oxidative stress which may induce many pathophysiological processes. Production of reactive oxygen and other reactive radical is a normal phenomenon in human metabolic processes. However, due to the deleterious nature of these reactive radicals, the body system under normal conditions has a natural mechanism with which these reactants are promptly removed or neutralized. Toxic elements like Pb, Cd, V and As which are known pro-oxidants that induce oxidative stress were also investigated. Due to a discrepancy between oxidative stress activity and antioxidant defenses, oxidative stress is linked to disruption of a variety of functions. Lipid peroxidation occurs in lipoprotein particles or membranes, resulting in a variety of products such as malondialdehyde and a number of hydroperoxides as well as hydroxides (Gonzalez-Fraguela *et al*., 2013). Oxidative damage to proteins and nucleic acids occurs when amino acids or nucleotides are modified, resulting in a variety of specific damage products (Pizzino *et al*., 2017; Pizzino; Cadet & Davies, 2017). This oxidative damage can cause cellular impairment and lead to development of many diseases and disorders, like ASD and CP.

The oxidative stress markers analyzed in this study are TPP, MDA, TAC, and OSI. These are markers of intracellular oxidative damage including those of the CNS. The results of markers of oxidative stress in this study showed that children with neurodevelopmental disorders (ASD and CP) had reduced total antioxidant capacity in comparison to neurotypical children. Total antioxidant capacity is a reflection of the ability of the body to withstand or neutralize the deleterious effects of oxidants, it is thus reflected by the oxidative index derived for the subject. Increased oxidative index observed in this study in children with CP may be an indication of a greater oxidative dysregulation in this group of participants in comparison to children with ASD that showed similar oxidative stress index with NT children. Similarly, reduced TAC observed in the two group of children may then be adduced to accumulation of oxidants which in this study were indicated by levels of TPP and MDA. Although there was

discordance in the TPP level in the two groups of children, however, MDA level was similarly increased in the two groups of children studied (ASD and CP) which may be an indication that the reduced TAC observed may be a consequence of MDA accumulation. Although TPP level was lower in ASD children relative to NT, the higher TPP level in children with CP may be responsible for the higher OSI observed in the latter group of children.

The main and most studied product of polyunsaturated fatty acid peroxidation is malondialdehyde (MDA). Lipids are the most active class of biomolecules among the various biological targets of oxidative stress. This aldehyde is a highly toxic molecule and since lipids are the largest molecules in the membrane, its association with DNA and proteins has been linked to several chronic diseases. In a review of antioxidants in health and diseases, Young and Woodside (2001), identified transition metals as major drivers of the formation of hydroxyl radical, a major oxidant in the human system. One of the transition metals investigated in this work was Cu. As previously stated, increased level of this essential element as seen inboth children with ASD and CP may have been precipitated by reduced Ca, Mg, Mn, Zn and Se levels all of which are known antioxidant metals preventing the accumulationof toxic metals and neutralizing ROS formed directly or by aiding the formation of catalase, super oxide dismutase and reduced glutathione (GSH). A dyshomeostasis of these natural antioxidative mechanism may have accentuated the process of genetic alteration aided by the presence of toxic elements like Pb, Cd, V and As.

Another plausible and possible aggravating phenomenon may be the known reduced appetite in children with ASD. This may have been precipitated by increased lipid peroxidation of the intestinal villi resulting in a reduction in absorption of nutrients including essential elements like Ca, Se, Mg, Zn and Mn with the attendant upregulation of the availability of toxic metals like Pb and Cd including conversion of Cu^{2+} to its pro-oxidation state (Phaniendra *et al.,* 2015). Cell membrane disruption, differences in membrane permeability and fluidity as well as oxidative damage to DNA, proteins and lipids may all result from an impaired antioxidant defense mechanism (Phaniendra *et al.,* 2015; Kurutas, 2016). Previous studies reported lower erythrocytes and plasma glutathione peroxidase and superoxidedismutase activities in ASD against the controls when measured in erythrocytes and plasma (Frustaci *et al.*, 2012). Also, previous study by Parellada *et al.* (2012) reported reduced TAS in ASD compared to healthy controls.

TPP and TAC concentrations were lower in patients with ASD and CP in this study, confirming previous findings. Reduced TAC/TAS may increase neuronal damage caused by normal or increased oxidants by impairing vulnerable individuals' defense mechanisms. Hence, results of oxidative stress markers in this study suggest that oxidative stress resulting from imbalance oxidant/antioxidant levels may lead to lipid peroxidation and neuronal DNA and RNA damage which will finally cause brain development impairment. This implies that oxidative stress could be a factor involved in the pathogenesis of ASD and CP disorders. This finding has been corroborated by many authors (Chen *et al.,* 2021; Omotosho *et al*, 2021; Liu *et al.,* 2022; Usui *et al.*, 2023).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1. SUMMARY

As the title of this project states, this extrapolatory-cross-sectional study investigated the possible effect of exposure to trace elements on oxidant/antioxidant profile and neurotransmitter activities in the pathogenesis of autism spectrum disorders. In executing this project, placenta transfer of essential and toxic metals to the developing foetus *in-utero* in exposed and non-exposed pregnant women in their third trimester to parturition was investigated. Findings from these subjects were then compared and extrapolated with biochemical indices from diagnosed clinical cases of autism towards establishing possible epigenetic involvement in the pathophysiology of autism spectrum disorders.

Summary of findings in this study are as below:

a. Toxic and essential elements were transferred from maternal circulation into the foetal system during baby's uterine life. Notwithstanding, Zn transported from the maternal circulation to the foetal system was not accessible at the cellular level to accomplish the required co-enzymatic activity, and thus may not be participating in the anticipated anti-oxidative processes.
b. Se and Mg may be the major antioxidant metals in the neuron *in-utero*; their deficiency may precipitate abnormal neurodevelopment in the newborns.
c. Uterine exposure may be the genesis of upregulations of toxic metals and down regulation of essential elements seen later in life as hallmarks in children with NDDs. This may initiate rapid depletion and/or displacement of essential elements (by toxic metals) especially in vulnerable subjects during active stages of neurogenesis in these children.
d. There were elevated levels of plasma Mn accompanied by significant reductions in plasma Ca, Se and Mg which may result in antioxidant/oxidant imbalance that could be a major factor accelerating the progression of ASD and CP.
e. Overactivity of neurotransmitter enzymes was also observed in the cord samples

indicating defined gross abnormality of neurogenesis from *in-utero*. The abnormal neuro transmitter activity may be a consequence of an imbalance in oxidant/antioxidant level which may be initiating excessive inhibition of neurotransmission by GABA resulting in over-excitation of the neurons typically manifesting in the known overactivity of children with ASD.

f. Increased MDA and reduced TAC was observed in cases in this study particularly in CP participants. This may be the basis of the increased oxidative stress resulting in oxidant/antioxidant imbalance thus facilitating the pathogenesis of NDDs particularly the CP. This may facilitate continuous lipid peroxidation resulting in DNA mutation and the RNA misconfiguration and neuronal damage in ASD.

6.2. CONCLUSION

The observed oxidant/antioxidant imbalance causes oxidative stress in due to increased toxic metal and reduced essential elements may initiate a continuous lipid peroxidation activity resulting in DNA and RNA misconfiguration in the neurons. The attendant deleterious effect on nerve impulse transmission may be the major pathogenesis of brain damage manifesting clinically as ASD and CP.

The rapid depletion/displacement of essential elements due to active metabolic activities as occurs in neurogenesis in these children may underscore the epigenetic theory linking the role of exposure to environmental/occupational toxicants in genetic modification as the basis of NDDs.

Hypotheses:

Based on findings in this project, the following are the possible hypotheses in the pathogenesis of ASD and CP:

1. Although Ca, Mg, Se, Mn, Cu and Zn levels are affected by placenta transfer of metals *in-utero*, antioxidation at the neuronal level is dependent on Se and Mg but not on Zn.

2. Imbalance in oxidant/antioxidant level initiate inhibition of GABA the resultant effect of which may be overactivity or underactivity of glutamate which plays a crucial role in the onset of neurodevelopmental disorders.

6.3. RECOMMENDATIONS

1. Pregnant women should be given supplements rich in Se, Ca and Mg but low in Cu. Essential elements particularly Zn and Cu may be recommended as therapeutic options in managing and reducing severity of ASD and CP.
2. Children with ASD and CP should be given supplements rich in Se and Vit. B6 to alleviate the symptoms and severity of the disorders.
3. Members of the public should be educated on the impact of the environmental and occupational toxicants on the brain developments.
4. Government should develop a policy to ensure our environment is free of many know toxicants that have deleterious effect on health particularly the developing brain.

6.4. CONTRIBUTIONS TO KNOWLEDGE

1. Zn ion transferred from the maternal circulation to the foetal system was not accessible at the cellular level to execute the essential co-enzymatic activity, and Se is the major antioxidant element in neuron, its deficiency in the baby *in-utero* may be a crucial determinant leading to improper neurodevelopment.
2. Elevated toxic metals and reduced essential elements predispose children to NDDs and are involved in the aetiopathogenesis of ASD and CP.
3. Neurotransmitters (Glutamate and GABA) played pivotal roles in aetiogenesis of ASD and CP.
4. Aetiopathogenesis of ASD and CP disorders can be linked to oxidative stress. Oxidant/antioxidant imbalance in ASD and CP may lead to lipid peroxidation, neuronal damage, DNA and RNA damage which can finally cause brain damage particularly in CP.
5. Male preponderance than female in children with ASD and CP is evident is this study
6. Haemoglobin genotype predispose children to neurodevelopment disorders and is involved in the aetiopathogenesis of ASD and CP.

6.5. SUGGESTIONS FOR FUTURE STUDY

i. Future study should focus on the analyses of essential elements in ASD and CP to be stratified by sex and age. The prevalence is more in a particular sex and many hypotheses have been put forward to explain the disparity.

ii. The main role of Selenium in the pathogenesis of NDDs is not fully understood. It was the only essential elements that was significantly different between ASD and CP, further investigation of its role at the cellular level especially *in-utero* may unravel some of the challenges in ASD and CP management.

iii. The actual mechanism of abnormal activity of glutamate resulting in abnormal neurotransmission genetic alteration induced by imbalance in oxidant/antioxidant level which may be the basis of the abnormal genetic alteration in ASD and CP should be further investigated.

iv. The involvement of heamoglobin genotype that predispose children with AA genotype to neurodevelopmental disorders (ASD and CP) should be investigated further at the molecular and genetic level.

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APPENDICES

INSTRUMENTATIONS

Inductively Coupled Plasma Mass Spectrometer – Agilent model 7500ce, equipped with an Octapole Reaction System (ORS). Inductively Coupled Plasma Spectromete – Perkin-Elmer, model 5300DV.

REAGENTS AND SOLUTIONSREAGENTS

a. Deionized water
b. Millipure water
c. Nitric acid (HNO_3)
d. Nitric acid (HNO_3) – concentrated.
e. Sodium Hydroxide (NaOH)
f. Mass spectrometer turning solution (10µg/L)

SOLUTIONS

25 – 50% NaOH solution

250 – 500 g NaOH weighed and dissolved in water to make 1L.2% NHO_3 solution: concentrated NHO_3 was diluted 1:50 with millipure water 20Ml/1L. the solution was stored in polypropylene bottles.

STANDARDS

Commercial standards were used in preparation of all the internal and elemental

Preparation of standard solutions

a. Internal standard (5000µg/L):

Volume equivalent to 500µg (500µl of 1000 mg/L) was measured and diluted to100lm with 2% HNO_3 and mixed.

b. Calibration standards –ICP-MS

Intermediate standards were prepared from the commercial standards solutions by diluting into a concentration of HNO_3.

Calibration standards were prepared by diluting prepared intermediate standards with 2% HNO_3 and enough 5000ug/L international standard to make final concentration of

5ug/L. (0ppb - Calibration blank and 5-50ppb concentrations were prepared).

c. Quality control standards

Quality control standards were also prepared from the commercially international standards

SAMPLE PREPARATION

All samples and materials were prevented from dust to avoid ntamination.

ASSAY PROCEDURE

Prepared samples and controls were put in microwave vessel liner that was clean. Separate vessels were used for each control of ICP-MS and ICP-EOS.

EXTRACTION PROCEDURE

a. Microwave Digestion:

5ml concentrated HNO_3 was added to each assembled vessel and placed in the microwave. The oven was programmed for 1200 Watts for power with Ramp time of 10 minutes, 180^{o}C final temperature. The hold and cool down time was10 minutes each. The samples were digested and transferred to fume hood for the vessel to equilibrate at room temperature. After which the vent was openedto atmospheric pressure.

b. Microwave Evaporation

Veseel liners were placed into the evaporation carousel and was taken to the microwave programmed for 600 Watts, Ramp time for 5 minutes. The final temperature was set to be 120^{o}C, with 3.5 minutes hold time and cool down timeof 10 minutes. Samples were evaporated and vessels were cooled at room temperature. The evaporation manifold was then flushed with deionised wate

c. Extraction Preparation

Extract was poured into tubes, rinsed and diluted for analysis. It was properly mixed by inverting after addition of standards. The mixture was then placed intoauto sampler for analysis

.

A sample set for ICP-MS and ICP-OES consists of

i. Reagent blank
ii. Negative control
iii. Positive control
iv. Samples

Calculations / identification

Instrument software was programmed to perform all necessary calculations. Results: was read in ppb and converted to other units.

ESTIMATION OF NEUROTRANSMITTERS

Group 2 samples were analysed for glutamine, glutamate and GABA using Melsin Human ELISA KIT

Microelisa stripplate	12*8strips
Standards (1 set)	0.3ml X6
Sample diluent	6.0ml X1
HRP-Conjugate reagent	10.0ml X1
20X Wash solution	25ml X1
Chromogen Solution A	6.0ml X1
Chromogen Solution B	6.0ml X1
Stop Solution	6.0ml X1
Closure plate membrane	2
User manual	1
Sealed bags	1

REAGENT PREPARATION

20×wash solution was dilute with Distilled water 1:20.

ESTIMATION OF OXIDATIVE STRESS MARKERS

Malondialdehyde(MDA) Estimation

REAGENTS:

30% of trichloroacetic acid(TCA). 0.75% OF thiobabituric acid(TBA).0.15M Tris-KCl buffer(pH7.4).

Preparation of Fox-2 Reagent:

9.8mg of Ammonium Ferrous sulphate was weighed and dissolved in 10 mls od 250mM.H_2SO_4) to produce 250µmol ferrous ion acid. The ferrous ion acid mixture was added to HPLC-grade methanol - 90ml. 7.6mg xylenol orange was added to the solution and stirred to produce the working solution. Blank solution contained only ferrous sulphate.

Standard used contained 100mM of hydrogen peroxide(H_2O_2).

PREPARATION OF FRAP REAGENT

1) 30mM acetate buffer of pH 3.6, sodium trihydrate 3.1g and glacial acetic acid-16ml were measured and mixed together, the sodium solution was made up to 1L with distilled water and stored at 4c.
2) 10mM 2,4,6-tripyridyl-s-trizone (TPTZ) in 40mM HCL; 0.031g TPTZ was weighed and dissolved in 10ml of 40mM HCL at 50˚c in water bath
3) 20mM $Fecl_3.6H_2O$;0.054g of $Fecl_3.6H_2O$ was weighed and dissolved in 10ml distilled water
4) At the time of use, the FRAP solution was the mixture of solutions prepared in(1),(2) and (3) above in ratio 10:1:1.
5) Standard contained 1000µM ascorbic acid.

APPENDIX

TELEGRAMS..... TELEPHONE.....

OYO STATE: THE PACESETTER

MINISTRY OF HEALTH

DEPARTMENT OF PLANNING, RESEARCH & STATISTICS DIVISION

PRIVATE MAIL BAG NO. 5027, OYO STATE OF NIGERIA

Your Ref. No. .....

All communications should be addressed to the Honorable Commissioner quoting

Our Ref. No. AD 13/ 479/____ July, 2015

The Principal Investigator,
Department of Chemical Pathology,
Faculty of Basic Medical Science,
College of Medicine,
University of Ibadan.
Ibadan.

Attention: Omotosho Ishiaq

Ethical Approval for the Implementation of your Research Proposal in Oyo State

This acknowledges the receipt of the corrected version of your Research Proposal titled: "Toxic Metals and Micronutrients Status as Biomarkers of Genetic Alteration in Children with ASD in Nigeria."

2. The committee has noted your compliance with all the ethical concerns raised in the initial review of the proposal. In the light of this, I am pleased to convey to you the approval of committee for the implementation of the Research Proposal in Oyo State, Nigeria.

3. Please note that the committee will monitor closely and follow up the implementation of the research study. However, the Ministry of Health would like to have a copy of the results and conclusions of the findings as this will help in policy making in the health sector.

4. Wishing you all the best.

OYO STATE RESEARCH ETHICAL REVIEW COMMITTEE
Signature & Date
MINISTRY OF HEALTH

Sola A... (Dr.)
Director, Planning, Research & Statistics
Secretary, Oyo State, Research Ethical Review Committee

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
College of Medicine, University of Ibadan, Ibadan, Nigeria.

Director: **Prof. Catherine O. Falade,** *MBBS (Ib), M.Sc, FMCP, FWACP*
Tel: 0803 326 4593, 0802 360 9151
e-mail: cfalade@comui.edu.ng lillyfunke@yahoo.com

UI/UCH EC Registration Number: **NHREC/05/01/2008a**

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Toxic Metals and Micronutrients Status as Biomarkers of Genetic Alteration in Children with Autism Spectrum Disorders (ASD) in Nigeria

UI/UCH Ethics Committee assigned number: UI/EC/15/0087

Name of Principal Investigator: **Dr. I. O. Omotosho**

Address of Principal Investigator: Department of Chemical Pathology,
College of Medicine,
University of Ibadan, Ibadan

Date of receipt of valid application: 23/03/2015

Date of meeting when final determination on ethical approval was made: **20/08/2015**

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and *given full approval by the UI/UCH Ethics Committee.*

This approval dates from **20/08/2015 to 19/08/2016.** If there is delay in starting the research, please inform the UI/UCH Ethics Committee so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the* UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC early in order to obtain renewal of your approval to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. *No changes are permitted in the research without prior approval by the* UI/UCH EC *except in circumstances outlined in the Code. The* UI/UCH EC *reserves the right to conduct compliance visit to your research site without previous notification.*

UI/UCH EC
20 AUG 2015
APPROVED

Professor Catherine O. Falade
Director, IAMRAT
Chairperson, UI/UCH Ethics Committee
E-mail: uiuchec@gmail.com

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Calcium and Magnesium Levels Are down Regulated in Nigerian Children with Autism Spectrum Disorder and Cerebral Palsy

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Abstract

Autism Spectrum Disorders (ASDs) and Cerebral Palsy (CP) are amongst the leading neurodevelopmental disorders in children worldwide causing diminished quality of life. Unlike CP caused by brain damage affecting muscle tone, movement and motor skills, equivocal report of different genes with varying loci as genetic malformation and genetic modulation by environmental factors have been the focus of attention in the aetiology of ASD. This study investigated levels of toxic metal (Pb) and macro elements (Ca and Mg) in blood of children with ASD and CP in Nigeria. 8 and 18 Children (aged 2 - 12 years) clinically screened for features of ASD and CP respectively by pediatric neurologist using DMS-IV classification along with 15 age-matched neurologically healthy ones as controls were recruited. Plasma levels of Ca, Mg and Pb were determined in the children using Induction Coupled Plasma Mass Spectroscopy (ICP-MS). Results were analyzed using students t-test. The gender difference was not significant in the children ($P = 0.216$) while developmental milestones' abnormalities (stable neck, sitting, crawling and walking) was significantly prevalent among CP children relative to ASD and normal children ($P= 0.003$, 0.003, 0.003 and 0.000 respectively); however, abnormality in talking was common in ASD and CP relative to normal children ($P = 0.000$). There was significant difference in educational background of ASD and CP parents relative to those of normal children ($P = 0.025$). Mean plasma calcium and magnesium levels was significantly reduced in children with ASD (7.90 ± 0.17 mg/dl, 2.44 ± 0.07 mg/dl) and CP (7.26 ± 0.31 mg/dl, 2.42 ± 0.08 mg/dl) in comparison to the controls (8.97 ± 0.20 mg/dl and 3.26 ± 0.16 mg/dl); ($P < 0.001$; $P < 0.000$ and $P < 0.002$; $P < 0.000$) respectively. However, mean lead levels in children with CP (10.38 ± 1.45 µg/dl) were significantly greater than in ASD (7.92 ± 1.30 µg/dl) and normal children

(6.83 ± 0.72 μg/dl) ($P < 0.433$; $P < 0.047$). Hypocalcaemia and hypomagnesaemia with concurrent plumbism (more pronounced in CP) was observed in children with ASD and CP in this study).

Keywords

ASD, CP, Calcium, Magnesium, Lead, Neurodevelopmental Disorders

1. Introduction

Autism Spectrum Disorders (ASD) describes a range of conditions classified as neurodevelopmental disorders in the fifth revision of the American Psychiatric Association [1]. It is a disorder characterized by social deficits and communication difficulties, stereotyped or repetitive behaviors and interests, sensory issues, and in some cases cognitive delays that manifest in early childhood [2]. The incidence of autism in the 90's was estimated at 1 in every 110 children [3]; however, recent studies have shown that the incidence is increasing globally with prevalence rates of 1.13% (1 in 88) in 2012 to 1.47% (1 of 68) in 2014 in Americans [4] [5] and 0.8% in 2011 to 2.3% in 2014 in Nigerians [6] [7] with a preponderance ratio of four males to a female. This global increase of ASDs prevalence cannot be fully explained in spite of advances in diagnostics because of sudden genetic shifts. There is therefore a growing consensus among scientists and clinicians that ASDs is probably caused from an interaction among genes, environment and the brain [8]. Several studies on environmental factors including those on identical twins have implicated either the exposure to toxins or lack of essential trace elements as important contributors in the pathophysiology of ASD [8]. Also, the plausibility of this has been corroborated by identifying roles of these elements and by the equivocal results of various genetic typing in children with ASD [9].

Cerebral palsy is a common developmental disability characterized by motor impairment and can present with general physical and mental dysfunction with worldwide incidence of 2 to 2.5 per 1000 live births [9]. It is a static neurologic condition resulting from brain injury that occurs before cerebral development is complete. Because brain development continues during the first two years of life, cerebral palsy can result from brain injury occurring during the prenatal, perinatal, or postnatal periods. The etiology of CP is therefore very diverse and multifactorial. The causes are diverse; they include congenital, genetic, inflammatory, infectious, anoxic, traumatic and metabolic. The injury to the developing brain may be prenatal, natal or postnatal. As much as 75% - 80% of the cases are due to prenatal injury with less than 10% being due to significant birth trauma or asphyxia [10].

Trace elements are the building blocks of our bodies which consist of macro elements that are required in milligram quantities and the micro elements that

are needed in micro and possibly nano quantities. They are required especially as coenzymes in many metabolic processes and also as building blocks for body and protein structure, fluid balance and many other processes in the body. They are keys for the health of every body system and functions [11]. Most of the essential trace elements are derived from diet; however, a good number are environmental contaminants/toxicants which inevitably find their way into the human system. Genetically, children with autism may be less able to detoxify toxic environmental agents, and this inability may predispose them to suffer neural damage consistent with autistic behavioral traits [12]. There have been suggestions that lack of essential minerals may cause many health problems, and a lack of them (or in some cases, an excess of them) could contribute to the etiology of ASD. This study was therefore designed to investigate the possible presence and role of abnormal levels of toxic (Pb) and macro elements (Mg and Ca) in the blood of children with ASD and those with CP.

2. Subjects and Methods

Recruitment: A total number of 41 participants were recruited for this pilot study. This comprised of eight (8) and eighteen (18) clinically diagnosed ASD and CP participants with mean age 5.25 ± 0.37 and 5.47 ± 0.81 respectively and fifteen (15) neurological healthy children with mean age 7.87 ± 0.89 as controls. Children with CP were included in this study to serve as positive control because CP is more prevalent and have also been well investigated. Distinguishing features of children with CP in respect of developmental milestones make the diagnosis clearer than ASD thus using CP as eliminating criterion while investigating neurodevelopmental disorders identification and hence recognition of ASD.

Selection: Selection of subjects was done by the Paediatric Neurologist based on above criteria and all autistic children were subjected to a full clinical child psychiatric evaluation for diagnosis of autistic spectrum disorder and exclusion of other psychiatric disorders according to Diagnostic and Statistical Manual of Mental Disorders based on DSM-IV-TR; DSM-5 classification. All the children recruited for the study received routine childhood vaccinations.

History of all children in the study was taken covering: parent socio-economic status, pregnancy history, attainment of developmental milestones, dietary history, and environmental exposure details. Past history of major childhood illnesses and immunizations were taken and clinical examination of all body systems with special emphasis on neurological examination was performed.

Ethical approval was obtained from the University of Ibadan/University College Hospital joint Ethical Committee.

Informed consent was obtained from each subject through their parents.

Inclusion criteria: Children clinically diagnosed as ASD and those as CP whose parents gave informed consent were recruited for the study.

Exclusion criteria: Children having liver or kidney disease, anemia, kwashi-

orkor or current treatment for iron deficiency, progressive neurological disorders, or epilepsy were excluded from the study.

Method

Blood collection: About 2 mls of venous blood sample was collected from each participant from the ante-cubital vein. Blood was carefully dispensed into lithium heparin bottles to avoid haemolysis. Blood samples were centrifuged at 2000r.p.m. for 10 minutes using Centaur 2-centrifuge (Fiston centrifuge, manufactured in England) to obtain plasma which was promptly separated into another clean plane bottle. All samples were kept frozen at −20˚C until they were ready for analysis.

Ca, Mg and Pb were determined using ICP-MS.

3. Results

Instrument software was used on the machine to calculate the results with the inclusion of appropriate standard and controls. The result was in part per billion (ppb); concentrations in ppb were converted to other units using appropriate conversion factor. Details of the results are in Tables 1-6.

Statistical analysis: Data obtained were revised, coded, tabulated, and analyzed using Statistical Package for Social Science (SPSS 20.0.1 for windows; SPSS Inc., Chicago, IL, 2016) according to the type of data obtained for each parameter.

Descriptive statistics: Mean standard error (±SE) was used for parametric numerical data, frequency and percentage for non-numerical data.

Analytical statistics: Student's t-test was used to assess the statistical significance of the difference between two study group means. ANOVA was used to assess the statistical significance of the difference between more than two study group means, chi-square test was used to examine the relationship between two qualitative variables, and fisher's exact test was used to examine the relationship between two qualitative variables when the expected count was less than 5 in more than 20% of cells. P value of 0.05 was considered significant.

Table 1 shows ASD group consisted of 6 boys (75%) and 2 girls (25%); CP consisted of 15 boys (83.3%) and 3 girls (16.7%) and the control group consisted of 8 boys (53.3%) and 7 girls (46.7%). Analysis of the questionnaires showed that 75.0% of ASD and 66.7% of CP were 1st child among siblings. Deductions from the result showed that parents of the children in the 3 groups belong to similar socio-economic class however fathers of CP children were least educated.

Table 2 shows profile of developmental milestones in the participants. 62.5% of ASD and 77.8% of children with CP were not talking; this value is significant when compared within the groups ($P < 0.000$). 38.9% of children with CP had unstable neck with delayed sitting and crawling ($P < 0.003$) while 55.6% were not walking. The other developmental milestones indicators showed that children with CP were significantly affected compared to children with ASD and none in

Table 1. Comparison of frequency distribution of socio-economic status within the group using chi-square.

VARIABLES	RESPONSE	ASD (N = 8)	CP (N = 18)	NC (N = 15)	X^2	P-value
SEX	MALE	6 (75%)	15 (83.3%)	8 (53.3%)	3.644	0.216
	FEMALE	2 (25%)	3 (16.7%)	7 (46.7%)		
CHILD BIRTH ORDER	1ST	6 (75.0%)	12 (66.7%)	8 (53.3%)	8.141	0.686
	2ND	2 (25.0%)	2 (11.1%)	2 (13.3%)		
	2ND	0 (0.0%)	0 (0.0%)	2 (13.2%)		
	3RD	0 (0.0%)	2 (11.1%)	3 (20.0%)		
	4TH	0 (0.0%)	1 (5.6%)	0 (0.0%)		
	5TH	0 (0.0%)	1 (5.6%)	0 (0.0%)		
MOTHER'S LEVEL OF EDUCATION	PE	0 (0.0%)	2 (11.1%)	0 (0.0%)	8.118	0.176
	PPE	1 (12.5%)	6 (33.3%)	4 (26.7%)		
	PSE	5 (62.5%)	9 (50.0%)	5 (33.3%)		
	PGE	2 (25.0%)	1 (5.6%)	6 (40.0%)		
FATHER'S LEVEL OF EDUCATION	PE	0 (0.0%)	1 (5.6%)	0 (0.0%)	14.395#	0.025*
	PPE	1 (12.5%)	5 (27.8%)	3 (20.0%)		
	PSE	5 (62.5%)	11 (61.1%)	3 (20.0%)		
	PGE	2 (25.0%)	1 (5.6%)	9 (60.0%)		

#Fisher's Exact Test, *significant at $P < 0.05$ (PE-Primary Education; PPE-Post Primary Education; PSE-Post Secondary Education; PGE-Post Graduate).

Table 2. Comparison of frequency distribution of developmental milestones within the group using chi-square.

VARIABLES	RESPONSE	ASD (N = 8)	CP (N = 18)	NC (N = 15)	X^2	P-value
STABLE NECK	YES	8 (100%)	11 (61.1%)	15 (100%)	9.390#	0.003*
	NO	0 (0.0%)	7 (38.9%)	0 (0.0%)		
SITTING	YES	8 (100%)	11 (61.1%)	15 (100%)	9.390#	0.003*
	NO	0 (0.0%)	7 (38.9%)	0 (0.0%)		
CRAWLING	YES	8 (100%)	11 (61.1%)	15 (100%)	9.390#	0.003*
	NO	0 (0.0%)	7 (38.9%)	0 (0.0%)		
WALKING	YES	8 (100%)	8 (44.4%)	15 (100%)	17.348#	0.000*
	NO	0 (0.0%)	10 (55.6%)	0 (0.0%)		
TALKING	YES	3 (37.5%)	4 (22.2%)	15 (100%)	23.487#	0.000*
	NO	5 (62.5%)	14 (77.8%)	0 (0.0%)		

#Fisher's Exact Test, *significant at $P < 0.05$.

controls. Deduction from these data showed that developmental milestones were either delayed or impaired in children with ASD and CP compared to control.

Table 3 shows that there were no significant differences in dietary and nutrition patterns of the three groups except for the nutritional supplement which revealed that 61.1% of children with CP were not taking nutritional supplement ($P < 0.001$). The level of education of parents of the children might have influenced administration of supplements towards enhancing the level of nutritional elements in the children.

Table 4 shows biodata variables among children with ASD, CP and controls.

Table 3. Comparison of frequency of nutrition/dietary history within the groups using chi-square.

VARIABLES	RESPONSE	ASD (N = 8)	CP (N = 18)	NC (N = 15)	X^2	P-value
FRUITS AND VEGETABLES	DAILY	4 (50.0%)	8 (44.4%)	5 (33.3%)	1.726#	0.849
	WEEKLY	3 (37.5%)	8 (44.4%)	9 (60.0%)		
	OCCASIONALLY	1 (12.5%)	2 (11.1%)	1 (6.7%)		
NUTRITIONAL SUPPLEMENTS	DAILY	3 (37.7%)	5 (27.8%)	0 (0.0%)	19.520#	0.001*
	WEEKLY	2 (25.0%)	0 (0.0%)	7 (46.7%)		
	OCCASIONALLY	2 (25.0%)	2 (11.1%)	4 (26.7%)		
	NONE USERS	1 (12.5%)	11 (61.1%)	4 (26.7%)		
SEA FOOD	DAILY	5 (62.5%)	11 (61.1%)	4 (26.7%)	7.829#	0.197
	WEEKLY	0 90.0%)	0 (0.0%)	2 (13.3%)		
	OCCASIONALLY	2 (25.0%)	3 (16.7%)	7 (46.7%)		
	NONE USERS	1 (12.5%)	4 (22.2%)	2 (13.3%)		
SUUPLEMENT IN PREGNANCY	DAILY	7 (87.5%)	18 (100%)	13 (86.7%)	4.233	0.252
	WEEKLY	1 (12.5%)	0 (0.0%)	1 (6.7%)		
	NONE USERS	0 (0.00%)	0 (0.0%)	1 (6.7%)		

#Fisher's Exact Test, *significant at P < 0.05.

Table 4. Comparison of biodata variables within the three group using anova (Mean ± S.E.).

VARIABLES	ASD (N = 8)	CP (N = 18)	CONTROL (N = 15)	F-VALUE	P-VALUE
Child's age (yrs)	5.25 ± 0.37	5.47 ± 0.81	7.87 ± 0.89	2.961	0.064
Mother's age at birth (yrs)	28.63 ± 1.59	30.50 ± 1.14	30.24 ± 1.29	0.575	0.567
Father's age at birth (yrs)	32.88 ± 1.60	37.22 ± 1.94	34.40 ± 1.097	1.089	0.347
Child's weight (kg)	19.31 ± 0.61	16.38 ± 1.81	19.67 ± 2.22	0.937	0.401
Child's birth weight (kg)	3.38 ± 0.07	2.86 ± 0.18	2.86 ± 0.23	1.591	0.217

There were no significant differences in children's age, mother's age at birth, father's age at birth, children's birth weight and children's weight between ASD and CP. It may therefore be said that there was no disparity in recorded bio data of the participants which might have influenced the study.

Table 5 shows the comparison of plasma levels of Mg, Ca and Pb in children with ASD and Control.

The mean plasma calcium and magnesium concentration in children with ASD were 7.90 ± 0.17 mg/dl and 2.44 ± 0.07 mg/dl respectively. These were significantly reduced when compared to 8.97 ± 0.20 and 3.26 ± 0.16 mg/dl in control ($P < 0.001$; $P < 0.002$ respectively). Although, the mean plasma lead levels in children with ASD was increased (7.92 ± 1.30 μg/dl) when compared with control (6.83 ± 0.72 μg/dl), the difference was not significant ($P < 0.433$). Hypocalcaemia and hypomagnasaemia maybe implied from this results.

Table 6 shows the comparison of plasma levels of Mg, Ca and Pb between children with CP and Control. The mean plasma calcium and magnesium

Tables 5. Comparison of biodata variables, trace and toxic metals in asd and control (Mean ± S.E.).

VARIABLES	ASD (N = 8)	CONTROL (N = 15)	t-value	P-value
Magnesium (mg/dl)	2.44 ± 0.07	3.26 ± 0.16	−3.669	0.001*
Calcium (mg/dl)	7.90 ± 0.17	8.97 ± 0.20	−3.616	0.002*
Lead (µg/dl)	7.92 ± 1.30	6.83 ± 0.72	0.799	0.433

*Significant at P < 0.05.

Table 6. comparison of biodata variables, trace and toxic metals in children with cp and control (Mean ± S.E.).

VARIABLES	CP (N = 18)	CONTROL (N = 15)	t-value	P-value
Magnesium (mg/dl)	2.42 ± 0.08	3.26 ± 0.16	−4.966	0.000*
Calcium (mg/dl)	7.26 ± 0.31	8.97 ± 0.20	−4.439	0.000*
Lead (µg/dl)	10.38 ± 1.45	6.83 ± 0.72	2.070	0.047*

*Significant at P < 0.05.

concentration in children with CP were 7.26 ± 0.31 mg/dl and 2.42 ± 0.08 mg/dl respectively. These were significantly reduced when compared to 8.97 ± 0.20 and 3.26 ± 0.16 mg/dl in control (P < 0.000; P < 0.000). Unlike in children with ASD, the mean plasma lead levels in children with CP (10.38 ± 1.45 µg/dl) were significantly increased when compared with the control (6.83 ± 0.72 µg/dl) (P < 0.047). Plumbism may be implied in children with CP with this result.

4. Discussion

Neurological disorders constitute a quarter of the top 20 health conditions leading to reported disability in about 1 billion people world-wide [13], ASD and CP are leading amongst these disorders; the prevalence rates is on the increase in developing countries just as in the developed countries. ASD, a known neurodevelopmental disorder, is characterized by dysfunctions in social, communications and repetitive behaviours. Its prevalence is reported to be 1.47% (1 of 68) in Americans [5] and 2.3% in Nigerian children [7]; the upsurge of the disorder in recent time has been of concern due to its undefined pathophysiology. There is therefore a growing interest in understanding the pathophysiology of the implication of environmental pollutants and micronutrients status in the aetiology of ASD. Cerebral palsy is a static neurologic condition resulting from brain injury that occurs before cerebral development is complete. Because brain development continues during the first two years of life, cerebral palsy can result from brain injury occurring during the prenatal, perinatal, or postnatal periods [12]. This study was designed to determine the plasma levels of Ca, Mg and Pb in these neurodevelopmental disorders (ASD and CP) towards assessing their possible contribution in the aetiopathogenesis of these disorders.

The significantly decreased level of magnesium observed in children with ASD and CP when compared with control in this study may be associated with gross abnormality in axon development and stabilization. Magnesium is an essential mineral that is necessary for the health of every cell in the body, including the proper functioning of brain and muscle cells. It is a macro element associated with axon development and stabilization. Amongst the known functions of axons in neural transmission is impulse carriage which ensures transmission of signals from one neuron to the other [14]. Although calcium is the element that regulates transmission of electrical signals in nerves, it's been reported that magnesium regulates entrance of calcium into the nerve cells [15]. Hence, a reduction in the level of Mg as observed in this study may be an impediment in the transmission of signals the manifestation of which may be the characteristic repetitive behaviour in children with ASD. Reports have shown that the earliest manifestations of magnesium deficiency are usually neuromuscular and neuropsychiatric disturbances [14]. Previous reports have also shown that magnesium protects cells from toxic metals and oxy-radical damage while assisting in the absorption and metabolism of vitamins C and E which are anti-oxidants important in cell protection [16]. Therefore, the consequence of a reduced level of Mg may include disturbance in the absorption of some of the other essential metals that may be needed for the basic functions of the neuron. Although, there are limited number of works on trace metals levels in neurodevelopmental disorders generally, most work on ASD were on hair and nail. In a similar study by Marlowe *et al.* (1983) [17], a significantly lower level of magnesium in the hair of autistic children was reported. However, the study by Melendez *et al.* (2013) [18] that reported a low serum magnesium level in ASD compared to reference value was similar to finding in this study.

On the other hand, the protective effect of Mg on the preterm neonatal brain particularly against CP may be inferred from studies on tocolysis. Previous studies showed that infants born to mothers given magnesium sulphate to prevent eclamptic seizures or as tocolysis showed a reduction in rates of cerebral palsy [19] [20]. The role of magnesium as a neuroprotective element in the development of CP may therefore be imperative; hence, the markedly reduced Mg level which was observed in children with CP and ASD may be prognostic of these neurodevelopmental disorders.

Also, the reduced Calcium level found in both children with ASD and CP in comparison to the controls may be as a result of reduced magnesium levels since both metals are known to be mostly interdependent in their activities [15]. Calcium regulation has been known to play a major role in neurodevelopment and synaptic plasticity [21]. It is crucial for the transmission of electrical signal along the nerves hence a reduction in calcium level may precipitate abnormality in signal transmission along the nerves and the developing neurons. The abnormality in the developing neuron will largely manifest as an abnormality in neurite outgrowth, a section of the neuron crucial for neural transmission; this may

explain the repetitive behavior in children with ASD. The tendency for this development may be due to the function of extracellular calcium-sensing receptor (CASR) present in the brain which is responsive to prevailing levels of extracellular calcium [22]. Thus, chronic or intermittent hypocalcemia in the patients may have a deleterious effect on neurite outgrowth and synaptogenesis, which may predispose these patients to neurodevelopmental alterations that ultimately manifest as neuropsychiatric sequela. Although the mechanism is unknown, previous workers have reported accumulating evidence for a causal role of calcium dysregulation in ASD. Such works clearly implicate calcium dysregulation as one of the most prominent biological factors in neurodevelopmental disorders risk when both genetic and environmental factors are considered [23] [24].

Lead level found in this study was increased in children with neurodevelopmental disorders compared to controls. Sources of exposure of the participants in this study to lead may be dust from the environment; this was amply demonstrated by the analysis of the questionnaire which showed that 37.5% (ASD) and 77.4% (CP) of the participants have their house situated on an untarred road. Tong and others (2010) reported that sources of exposure to lead include leaded gasoline, leaded paint, dust and soil contaminated with lead, water carried in lead pipes, industrial emissions or occupational exposures [25]. Lead (Pb^{2+}) has been shown to induce cognitive and behavioral deficits in children and adult with elevated levels of exposure resulting in a distinct neurological effects, with different brain targets and modes of action [26] [27]. Children are more vulnerable to air-borne metallic lead than adults since their respiration and metabolic rates are higher than in adults [13] (WHO, 2011), the toxic effect is thus more pronounced in children where it interferes with the normal development of a child's brain and nervous system. Previous studies done on hair reported increased lead levels in ASD compared to controls [9] [12] [27] just as it has been observed in this study that lead levels in ASD was higher than in control. The higher preponderance of Pb level in children with CP in comparison to those with ASD may be inferred from the residential locations of the children as deduced from the questionnaire. It could be seen that a greater percentage of children with CP lived nearer untarred road. Overall, exposure to Pb observed in this study agreed with other studies [28] [29] [30] [31]. They all reported increased blood lead levels in children with ASD. Secondly, calcium has also been reported to be selectively displaced by lead when the latter is in excess [32]; hence, the increased lead level in this study may be precipitated by the hypocalcaemia earlier observed in children with ASD and CP in this study. Nutritionally, children with ASD displayed lower levels of the essential elements calcium, copper, magnesium and selenium [33]. Since autistic children display poor eating habits; the low tissue levels may be explained by an inadequate nutritional intake.

The pathophysiology of ASD may also be due to increased blood lead level observed in this study which has been implicated in disturbance in the redox ac-

tivity of the brain. This is because increased blood lead level has been reported to have a variety of effects on synaptic mechanisms and structures resulting in formation of reactive oxygen species, speeding mitochondrial self-destruction through formation of the permeability transition pore, and priming activation of programmed cell death processes [31]. Increased blood level also interferes with neurotransmitter release, disrupting the function of GABAergic, dopaminergic, and cholinergic systems [31]. It has also been suggested that children with ASD show greater concentration of potentially toxic metal Pb in tissue; this may be the result of a greater ability to accumulate toxins, which in turn leads to an alteration of biochemical processes. It may therefore be inferred that children with neurodevelopmental disorders particularly ASD and CP may have problems with the chemical pathway that allows them to detoxify metals [15].

5. Conclusion

Based on the results of the present study it may be concluded that the levels of Mg and Ca which are essential macro metals were reduced and toxic metal (Pb) was increased in neurodevelopmental disorders as manifested in children with ASD and CP. Comparatively, there was an inverse relationship between the level of toxic metals and the essential macro elements; the ratio is more severe in CP than in ASD. The study thus suggests that the toxic effect of lead and reduced levels of the macro elements Ca and Mg may be prognostic of these neurodevelopmental disorders.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Environmental Exposure to Lead, Vanadium, Copper and Selenium: Possible Implications in the Development of Autism Spectrum Disorders

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Abstract

Human exposure to toxic metals is on the increase especially in the developing world; this is compounded by the almost unavoidable application of the metals domestically and industrially and their implication in several genetic defects, aging and some chronic illnesses including Autism Spectrum Disorders (ASD). This study investigated the concentration of toxic metals (Pb and V) and micro-essential elements (Cu and Se) in children with ASD and controls in Nigeria towards establishing their possible associations with the aetiopathogenesis of ASD. Eight children clinically diagnosed by Paediatric Neurologist and Child Psychiatrist for ASD using DMS-IV and fifteen apparently healthy children (age range 2 - 12 years) were recruited as cases and controls respectively. Plasma levels of Pb, V, Cu and Se were analyzed using Induction ICP-MS. Results were analyzed using students t-test. The mean plasma lead and vanadium levels were (7.92 ± 1.30 µg/dl; 1.07 ± 0.22 µg/dl) and (6.83 ± 0.72 µg/dl; 2.59 ± 0.48 µg/dl) in children with ASD and in controls respectively. The result showed that blood lead level in ASD was slightly increased but not significant when compared with control ($p < 0.433$). On the other hand, plasma vanadium concentration in ASD was significantly reduced (1.07 ± 0.22 µg/dl) when compared with control (2.59 ± 0.48 µg/dl) ($P < 0.038$). Mean plasma copper was similar in all participants (1.98 ± 0.13, 2.23 ± 0.12) but selenium concentrations were significantly reduced (0.37 ± 0.05 mg/L; 0.57 ± 0.02 mg/L) in ASD relative to controls respectively. Given the physiological functions of vanadium and selenium, the observed reduced levels of the two elements in children with ASD may account for the speech

and other neurological dysfunctions of the brain in ASD.

Keywords

Toxic and Essential Metals, Autism Spectrum Disorder, Children, Aetiopathogenesis

1. Introduction

Autism Spectrum Disorders (ASD) are a heterogeneous group of neurodevelopmental disorders that are behaviourally defined and characterized by impairments in communication and social interaction along with restrictive and repetitive behaviours beginning in infancy and toddler years [1] [2]. Several reasons have been proffered as the pathophysiology of ASD, however, contribution of industrial chemicals and metallic pollutants widely disseminated in the environment have been suggested as important contributors to the development of this condition [3] [4]. The vulnerability of the developing human brain in-utero has also lent credence to its prevalence among children. Recently, interaction between genes and the toxicants has been suggested as basis of genetic modulation and the development of ASD [5]. Thus, due to this complex pathophysiology of ASD, many authors are of the opinion that both genetic and environmental factors (and their interactions) are strongly linked in the development of the disorders [6]. The possibility of this assertion has been heightened by the recent increase in prevalence of the disorder worldwide.

Specifically, in recent times, research and clinical studies have implicated physiological and metabolic systems that transcend specific organ dysfunction, such as immune dysregulation, inflammation, impaired detoxification, impaired redox regulation/oxidative stress and energy generation/mitochondrial systems [7] [8]. These imply that ASD may arise from, or at least involve, systemic physiological abnormalities rather than being a purely central nervous system disorder in a subset of individuals with ASD [9]. It is in this respect that the involvement of trace and toxic metals (which in a developing economy like ours are inevitable pollutants) and their interaction with gene development especially in subjects that are actively or passively exposed to them become important.

Trace elements are chemical micronutrients which are required in small amounts but play a vital role in various physiological and metabolic processes occurring within living tissues [10] [11]. There are threshold levels for most of these trace elements; hence, their deficiency or excessive accumulation may cause serious changes in the body leading to disruption of important enzyme activities [12]. The immature blood-brain barrier, neuronal growth migration and myelination processes that occur on a specific and rapid schedule in a developing foetus may largely facilitate development of ASD in children.

In pregnancy, excessive exposure to heavy metals and some trace elements has

been shown to be harmful to the developing fetus and may be harmful to the human nervous system, even at low levels [13] [14]. Also, several studies have reported that trace metals could easily cross the placenta and affect cognitive development [15] despite its function as a barrier protecting the fetus from toxic metal exposure. The possible interaction between these toxic metals either in modulating the nutritional function of the essential metals or on genetic pairing remains issues in the aetiopathogenesis of ASD. Until recently, the study of potential environmental toxicant contributions to the development of ASD has been generally "neglected" [16], hence the need for a clearer look at their contribution to the development of ASD especially with the gradual increase in the prevalence of this disorder.

2. Subjects and Methods

Recruitment: A total number of 23 participants were recruited by convenience sampling method for this pilot study between August and November 2015. This comprised of eight (8) children clinically diagnosed for ASD with mean age 5.25 years ± 0.37 and fifteen (15) apparently healthy children with mean age 7.87 years ± 0.89 as controls. All children recruited for this study received routine childhood vaccinations.

Selection: Paediatric Neurologist and Child Psychiatrist clinically selected the subjects after proper evaluations and diagnosis according to Diagnostic and Statistical Manual of Mental Disorder DMS-IV-DR; DMS-5 classification. Children and their parent's history were taken.

Ethical approval was obtained from the UCH/UI joint Ethical Committee.

Informed consent was obtained from each subject through their parents.

Inclusion criteria: Clinically diagnosed children with ASD that their parents gave informed consent were recruited for the study.

Exclusion criteria: Participants that were suffering from liver or kidney disease, anemia, or current treatment for iron deficiency, progressive neurological disorders, or epilepsy were excluded from the study.

Method: History of all children in the study was taken covering: parent socio-economic status, pregnancy history, developmental milestones, dietary history, and environmental exposure factor. History of major childhood illnesses and immunizations were taken and clinical examination of all body systems with special emphasis on neurological examination was performed by a Neuro-paediatrician. All autistic children were subjected to a full clinical child psychiatric evaluation for diagnosis of autistic spectrum disorder and exclusion of other psychiatric disorders according to Diagnostic and Statistical Manual of Mental Disorders based on DSM-IV-TR; DSM-5 [17] [18].

Blood collection: About 2mls of venous blood was collected from each participant from the ante-cubital vein. Blood was carefully dispensed into lithium heparin bottles to avoid haemolysis. Blood samples were centrifuged at 3000 r.p.m. at room temperature for 10 minutes using Centeur 2-centrifuge (Fiston

centrifuge, manufactured in England) to obtain plasma which was promptly separated into another clean plane bottle. All samples were kept frozen at -20°C until they were ready for analysis. They were analyzed for Cu, Se, V and Pb using Induction Coupled Plasma-Mass Spectrometry (ICP-MS).

Statistical analysis: Appropriate in-built software in the instrument was used to calculate the result of the analysis with inclusion of standards and controls. The results were converted to SI units from part per billion (ppb) using appropriate conversion factors.

Data were reviewed, coded, tabulated and analyzed using statistical package for social science (SPSS 20.0)

Mean standard error (MEAN ± S.E) was used to express descriptive statistics of the results while student t-test was used to access the statistical significance of the difference between the two groups. P value of 0.05 was regarded significant.

3. Results

Table 1 showed gender percentage distribution; 6 boys (75%) and 2 girls (25%) constituted the ASD group while 8 boys (53.3%) and 7 girls (46.7%) constituted the neurotypical (control) group. The result showed that 6 (75.0%) of the ASD group were 1st born among the siblings. It was revealed from the results that the parents of the two groups belong to the same socio-economic status.

Table 1. Comparison of % gender distribution and socio-economic grouping of the two groups.

Variables	Response	ASD (N = 8)	NC (N = 15)	X^2	p-Value
Sex	Male	6 (75%)	8 (53.3%)	1.028	0.311
	Female	2 (25%)	7 (46.7%)		
Child birth order	1ST	6 (75.0%)	8 (53.3%)	2.008	0.366
	2ND	2 (25.0%)	2 (13.3%)		
	2RD	0 (0.0%)	2 (13.2%)		
	3RD	0 (0.0%)	3 (20.0%)		
	4TH	0 (0.0%)	0 (0.0%)		
	5TH	0 (0.0%)	0 (0.0%)		
Mother's level of education	PE	0 (0.0%)	0. (0.0%)	1.840	0.399
	PPE	1 (12.5%)	4 (26.7%)		
	PSE	5 (62.5%)	5 (33.3%)		
	PGE	2 (25.0%)	6 (40.0%)		
Father's level of education	PE	0 (0.0%)	0 (0.0%)	4.214	0.122
	PPE	1 (12.5%)	3 (20.0%)		
	PSE	5 (62.5%)	3 (20.0%)		
	PGE	2 (25.0%)	9 (60.0%)		

Legend: PE—Primary Education, PPE—Post Primary Education, PSE—Post Secondary Education, PGE—Post Graduate Education.

Table 2. Comparison of biodata variables in ASD and control (mean ± S.E.).

Variables	ASD (N = 8)	Control (N = 15)	t-Value	p-Value
Child's age (yrs)	5.25 ± 0.37	7.87 ± 0.89	−2.723	0.050
Mother's age at birth (yrs)	28.63 ± 1.59	30.24 ± 1.29	−1.028	0.316
Father's age at birth (yrs)	32.88 ± 1.60	34.40 ± 1.097	−0.990	0.333
Child's weight (kg)	19.31 ± 0.61	19.67 ± 2.22	0.240	0.814
Child's birth weight (kg)	3.38 ± 0.07	2.86 ± 0.23	1.629	0.118

*significant at $p < 0.05$.

Table 3. Comparison of trace and toxic elements levels in ASD and control (mean ± S.E.).

Variables	ASD (N = 8)	Control (N = 15)	t-Value	p-Value
Copper (mg/L)	1.98 ± 0.13	2.23 ± 0.12	−1.290	0.211
Selenium (mg/L)	0.37 ± 0.05	0.57 ± 0.02	−5.316	0.000*
Lead (µg/dl)	7.92 ± 1.30	6.83 ± 0.72	0.799	0.433
Vanadium (µg/dl)	1.07 ± 0.22	2.59 ± 0.48	−2.219	0.038*

*significant at $p < 0.05$.

Table 2 showed the biodata of participants. There were no significant differences in the children's age, mother's age at birth, father's age at birth, children's birth weight and children weight between ASD and control. It may be said that there were no disparities in recorded biodata of the two groups.

Table 3 showed the comparison of levels of Pb, V, Cu and Se in ASD and control groups. The result showed there were no significant differences in the mean levels of Cu (1.98 ± 0.13 mg/L; 2.23 ± 0.12) and Pb (7.92 ± 1.30 µg/dl; 6.83 ± 0.72 µg/dl) in ASD and controls respectively ($p < 0.211$; $p < 0.433$). However, Se level was significantly reduced in ASD when compared with the control (0.37 ± 0.05 mg/L and 0.57 ± 0.02 mg/L) respectively ($p < 0.000$). Also, plasma vanadium level was significantly reduced in ASD compared to controls (1.07 ± 0.22 µg/dl and 2.59 ± 0.48 µg/dl) respectively ($p < 0.038$).

4. Discussion

Autism spectrum disorders (ASDs) are a heterogeneous group of neurodevelopmental disabilities that can cause significant social, communication and behavioral challenges that are characterized by impairments in communication and social behaviors beginning in infancy and toddler years [1]. It has a prevalence of 1.47% in Americans [19] and 2.3% in Nigerian children [20]. ASD has been reported to account for 7.7 million disability adjusted life years in 2010 and was the leading mental cause of disability in children under five in terms of years lived with disability [21]. While the exact etiology of ASD remains unknown, novel technologies and large population-based studies have provided new insight into the risk characteristics of ASD and the possible role of environmental factors in its etiology [22] [23].

The study was designed to determine the plasma levels of V, Cu, Se and Pb in ASD and based on their biochemical roles, to compare with controls in order to assess their possible contribution to the disorder. The increased blood Pb level (though not significant) found in this study may be pathognomonic of the disease. This may be the basis of the impaired cognitive and behavioural deficits characteristic of this condition. This may be facilitated by the increased sensitivity of the brain to lead exposure [24]. Lead has been severally reported to displace other elements like calcium and magnesium from most of their reactive sites through several processes. The pathological effect especially when present in a prolonged and excessive amount may contribute to the development of ASD. Its effect inducing cognitive and behavioral deficits in children and adult resulting in a distinct neurological effect, with different brain targets and modes of action has been severally documented [25] [26]. Hence, the possible deleterious effect of air-borne lead in children compared to adults because of the higher respiratory and metabolic activities of the former may have also facilitated the development of ASD in these children. Previous studies done on hair lead level reported increased lead levels in ASD compared to controls [27] [28] [29] just as it has been observed in this study that blood lead level in ASD was higher than in controls. This was also in agreement with other studies that reported increased blood levels in ASD [30] [31]. Secondly, since calcium has also been reported to be selectively displaced by lead when the latter is in excess; the increased lead level in this study may be precipitated by the hypocalcaemia observed in children with ASD which was reported earlier by this team [30]. Nutritionally, children with ASD have been reported to have low poor eating habits with attendant low nutritional compliments. It may therefore be possible that reduced essential elements level in ASD children may also exacerbate the development of autism especially in an environmentally prone setting.

The increased blood lead level observed in this study may also be implicated in a disturbance in the redox activity of the brain. This is because increased blood lead level has been reported to have a variety of effects on synaptic mechanisms and structures resulting in formation of reactive oxygen species, speeding mitochondrial self-destruction through formation of the permeability transition pore, and priming activation of programmed cell death processes [32] [33]. Lead has also been shown to interfere with neurotransmitter release, disrupting the function of GABAergic, dopaminergic, and cholinergic systems [32]. However, there have been other reports suggesting that there is an inherent higher susceptibility of certain children to lead toxicity relative to others. It may therefore be inferred that children with ASD may have problems with the chemical pathway that allows them to detoxify metals to alleviate different cluster of autistic symptoms. Although, this is equivocal, evidence shows that autistic children show an increased build-up of toxins which may not arise simply from excessive exposure but from a marked inability to process and eliminate toxins from the body. Such a mechanism could lead to accumulation of toxic heavy metals and chemical toxins with the attendant risk of the accumulation leading

to increase in free radical activity in the body [34]. Studies on this element have been very scanty especially in humans.

The major source of exposure to vanadium for the general population is food. The major report on this element has been on its insulin-like action in-vivo. Vanadium in the form of vanadate and vanadyl sulfate has been shown to improve the effect of insulin in diabetic animals; artificially induced diabetes in rats can be reversed by vanadate [35]. Large doses also affect serum fat and cholesterol levels, though more research in this area is needed. Biochemically, the possibility that vanadium may play a role in the regulation of Na^+/K^+-exchanging ATPase, phosphoryl-transfer enzymes, adenylate cyclase and protein kinases has been reported. The possible role of the vanadyl ion as an enzyme cofactor and its roles in hormone, glucose, lipid, bone and tooth metabolism have also been discussed [36]. No specific biochemical function has yet been identified for vanadium in higher animals. However, the recent discovery of vanadium-activated enzymes in lower forms of life lends credence to the view that vanadium has similar roles in higher animals. In this study, plasma level of this essential element was found to be low in children with ASD. The level was significantly low in comparison to that of control children. Based on what has been reported in lower animals, the essentiality of this element for normal growth and development is not in doubt. Hence, the observed reduced level in children with ASD may be associated with underdevelopment of the cognitive functions of the brain in this group of neurodevelopmental disorder. Biochemically, this may be due to its role in Na^+/K^+ ATPase and Phosphoryl enzyme transfer activities which are all linked to calcium homeostasis making growing children more vulnerable especially in sensitive organs like in their brain.

Copper is one of the micro elements included in this study. It was found that the Cu level in ASD was reduced (though not significantly) when compared to control. Copper is a trace element essential for body cellular functions. Characteristically, Cu could act as an oxidant or as an antioxidant depending on the concentration and the prevailing circumstance [37]. Functionally, copper promotes neurological activities and plays a role in antioxidant defense system of the body. Cu deficiency is known to affect the central nervous system. Specifically, information from animal studies suggests that Cu deficiency may be a strong factor in the pathogenesis of Parkinson's disease in humans especially since the level of one of the neurotransmitters (dopamine) was low in both Cu deficient animals and in patients with Parkinson's. Cudeficiency has also been implicated in Menke's disease which is a neurodevelopmental inherited disease [38]. Cu deficiency has also been shown to be exacerbated by the increased presence of elements like lead and cadmium. Hence, since Cu is largely derived from the diet, deficient nutritional status occasioned by reduced appetite in ASD may have also contributed to the progression of the pathological symptoms of ASD especially in a vulnerable environment. The observed reduced Cu level in ASD children in this study may have largely been accentuated by the above prevailing factors resulting in the progression of the pathological symptoms of the

disorder [39]. Although findings in this study were contrary to previous studies that reported elevated copper levels in ASD compared with control [40] [41] [42], this work was in agreement with the report of Craciun *et al.* that reported reduced Cu level in children with ASD [43].

Selenium is an essential trace metal needed in the production of antioxidants enzymes. As Selenoproteins, they are essential for brain development, redox control, and preventing and reversing oxidative damage in the brain and neuroendocrine tissues [44]. This important redox property of Se may have been compromised by the reduced Se level observed in ASD children in this study. The reduced selenium level found in this study may have contributed to the pathophysiology of ASD especially since glutathione is a very prominent antioxidant in the neuroendocrine tissues of the brain. Glutathione is known to be a major redox buffer in the trans-sulfuration pathway in the neuroendocrine tissue [45], this process ensures replication of the redox form of the antioxidant which contributes to the transmission of impulse along the nerves. It may therefore be inferred that a reduced level of Se greatly hampers this mechanism and may largely account for some of the neuro-behavioural changes associated with ASD. Control of intracellular oxidative tone and findings of increased oxidative damage in children with ASD may be indicative of disruptions of selenoenzyme activities resulting from reduced selenium levels. This finding agreed with previous studies that reported reduced Se levels in analysis done on hair and red blood cells in their studies [46] [47] [48].

5. Conclusion

The hallmark of ASD in children in this environment may be indicated by a deficiency in the essential trace elements-V, Cu and Se with a concurrent increase in Pb especially in environmentally vulnerable children. Abnormality in the levels of these elements may be good biomarkers of ASD especially in environmentally vulnerable children with or without the presence of the known clinical symptoms of ASD.

6. Limitation

The small sample size was a limitation in this study, a larger sample size may reveal more. Also, control of environmental pollution is difficult in this part of the world; this may be due to level of public health awareness.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Oxidative stress indices in ASD children in Sub-Sahara Africa

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Abstract

Background: The pathogenesis of autism spectrum disorder (ASD) remains a medical challenge even in the developed world. Although genetics and epigenetic factors have been variously indicted as major causes of the disorder, development of oxidative stress especially in the formative years of children has equally gained prominence as an etiological basis of the disorder. Oxidative stress is characterized by the production of excessive amounts of free radicals, decreased levels of antioxidants with the attendant imbalance in oxidant/antioxidant ratio. This study was designed to determine the levels of essential metals [magnesium (Mg), zinc (Zn), and copper (Cu)] and toxic metal, lead (Pb), and generation of oxidative stress by their abnormal interaction.

Method: Twenty-five children clinically diagnosed for ASD according to DSM-IV-TR and 25 neuro-typical (NT) children (controls), (aged 5.96 ± 1.40 years and 6.18 ± 2.59 years respectively) were recruited for this study. Essential and toxic metals were analyzed using induction-coupled plasma-mass spectrometry (ICP-MS); oxidative stress markers [malondialdehyde (MDA), total plasma peroxidase (TPP), and total antioxidant capacity (TAC)] were determined using appropriate biochemical methods. Oxidative stress index (OSI) was calculated.

Results: The levels of TPP and TAC were significantly reduced while MDA was higher in ASD compared to NT. Although OSI was higher in ASD, the difference was not significant. Pb (lead) concentration was significantly increased while Mg, Zn, and Cu levels were reduced significantly in ASD compared to NT. A significant negative correlation between Mg and OSI ($r = -0.438$; $p = 0.029$) was observed in NT.

Conclusion: Reduction in Zn and Mg levels with a concurrent increase in Pb in children with ASD in this study may be the basis of inadequate TAC manifesting as increased MDA and reduced TPP levels. The attendant imbalance in oxidant/antioxidant ratio may result in abnormality in neuronal transduction leading to the abnormal cognitive and speech functions characteristic of ASD.

Keywords: Essential and toxic metals, Oxidative stress, Autism spectrum disorder, Imbalance in oxidant/antioxidant ratio

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is becoming increasingly prevalent. ASD is a heterogeneous group of neurodevelopmental disorders characterized by impairments in verbal and non-verbal expressive speech, deficits in social interaction and hyper-focused repetitive behaviors [1, 2]. The causes and pathophysiology of ASD are not fully understood. There is a general agreement that ASD could result from interaction between genetic and environmental factors with oxidative stress as a potential link [3]. Oxidative stress has recently been linked to the etiology of this disorder along with multiple genetic and environmental factors [4, 5]. Oxidative stress is characterized by the production of excessive amounts of free radicals, decreased levels of antioxidants, or both. Excessive production of free radicals or impaired antioxidant mechanism may cause oxidative stress which may induce several

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pathophysiological processes. Cellular antioxidant defense mechanism prevents the generation of free radicals and inactivates them after generation. Impaired antioxidant defense mechanism can result in cell membrane damage, alteration in membrane fluidity and permeability, and oxidative changes in proteins, lipids, and DNA [6]. Several environmental factors like heavy metals particularly Pb have been implicated in ASD. Understanding the levels of anti-oxidative/oxidative status in children with ASD may assist in clarifying the biochemical mechanisms underlying the pathophysiology of these disorders. That the pathophysiology of ASD involve oxidative stress resulting from exposure to heavy metals as environmental pro-oxidants has been reported [6]; however, there are limited or no information on this aspect of the disorder in sub-Sahara Africa. This write-up, therefore, examines the impact of exposure to some toxic and essential metals on levels of oxidative stress markers/indices in the development of ASD using black African children as a model.

Subjects and method

A total number of 50 participants were recruited into this study. This comprised 25 children clinically diagnosed for ASD (5.25 ± 0.37 years) as cases by specialists in child neurology and child psychiatry after proper evaluations and diagnosis using Diagnostic and Statistical Manual of Mental Disorder (DMS-IV-TR) classification and 25 neurotypical (NT) children (6.18 ± 2.2.59 years) as controls. The control were recruited from friends, colleagues, and neighbors, unrelated to the case group. All participants recruited for this study received routine childhood vaccinations/immunization.

Ethical approval

Ethical approval was obtained from the UCH/UI joint Ethical Committee (UI/EC/15/0087) and Oyo State Ministry of Health Ethical Board. Informed consent was obtained from each participant by proxy.

Inclusion criteria

Clinically diagnosed children with ASD that their parents gave informed consent were recruited for the study.

Exclusion criteria

Participants that were suffering from liver or kidney disease, anemia, or current treatment for iron deficiency, progressive neurological disorders, or epilepsy were excluded from the study. None of the participants was on psychotropic drug.

Blood sample was collected randomly from each participant from the ante-cubital vein. Blood was carefully dispensed to avoid hemolysis into lithium heparin bottles. Blood samples were centrifuged at 2000 r.p.m. at room temperature for 10 min using centeur 2-centrifuge (Fiston centrifuge, manufactured in England) to obtain plasma.

All samples were kept frozen at − 80 °C and were later analyzed for metals and oxidative stress markers.

Essential and toxic metals were analyzed using ICP-MS. Level of malondialdehyde (MDA) was estimated using the method of Adam-Vizi and Seregi [7], Total plasma peroxidase (TPP) was estimated using Fox-2 reagent as described by Uma Devi et al. [8] with minor modifications by Harma et al. [9] and total antioxidant capacity (TAC)] was determined using ferric reducing antioxidant powder (FRAP) reagent as described by Benzie and Stram [10] while oxidative stress index (OSI) was calculated as the ratio of total peroxide to the total anti-oxidant capacity.

Statistical analysis was done using Statistical Package for Social Science (SPSS 20. 0.1 for windows; SPSS Inc., Chicago, IL, 2016).

Descriptive statistics

Mean standard error (± SEM) was used for parametric numerical data, frequency, and percentage for non-numerical data.

Analytical statistics

Student's *t* test was used to assess the statistical significance of the difference between two study groups means.

ANOVA test was used to assess the statistical significance of the difference between more than two study groups means.

Chi-square test was used to examine the relationship between two qualitative variables. Fisher's exact test was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells.

P value of 0.05 was considered significant.

Results

Table 1 is a summary of the biodata variables between the autism spectrum disorders and neuro-typical children

The results showed no significant differences in all the biodata variables except the birth weight that showed significantly increased birth weight for ASD compared to NT ($p = 0$). Although, there was a difference in birth weight of cases and controls, the two groups were comparable based on other biodata obtained through structured questionnaire.

Also, the summary of mean ± SEM of essential trace metals and toxic metal between ASD and NT showed significant differences in all the biochemical parameters. Mg, Zn and Cu were significantly reduced in ASD compared to NT ($p < 0.000$; $p < 0.000$ and $p < 0.049$) respectively. Zn/Cu ratio was also significantly reduced in

Table 1 Comparison of Biodata and Biochemical Variables Between ASD and NT Groups Using Student T- Test

VARIABLES	ASD ($N = 25$)	NT ($N = 25$)	*t*-VALUE	*P*-VALUE
Child's Age (yrs)	5.96 ± 1.45	6.18 ± 2.2.59	− 0.370	0.713
Mother's Age at Birth (yrs)	26.68 ± 2.69	27.96 ± 2.70	−1.680	0.100
Father's Age at Birth (yrs)	31.72 ± 2.98	32.32 ± 3.86	−0.615	0.541
Number of Siblings	1.72 ± 0.79	1.60 ± 0.91	0.497	0.622
Child's weight (kg)	19.64 ± 3.99	19.00 ± 5.18	0.490	0.627
Mg (mg/dl)	2.53 ± 0.46	3.13 ± 0.43	−4.830	**0.000***
Zn (μg/dl)	222.3 ± 63.8	438.5 ± 185.5	−5.511	**0.000***
Cu (μg/dl)	4.32 ± 1.02	4.88 ± 0.94	−2.020	**0.049***
Zn/Cu	55.31 ± 22.04	92.29 ± 44.57	−3.719	**0.001***
Pb (μg/dl)	9.49 ± 4.04	5.43 ± 2.04	4.483	**0.000***
Total Plasma Peroxidase (TPP)	105.9 ± 2.3	110.4 ± 7.9	−2.679	**0.010***
Total Antioxidant Capacity (TAC)	280.2 ± 34.4	303.8 ± 33.1	−2.468	**0.017***
Malondialdyde ($\times 10^{-5}$) (MDA)	2.27 ± 0.23	1.42 ± 0.13	16.127	**0.000***
Oxidative Stress Index (OSI)	0.38 ± 0.05	0.37 ± 0.05	1.083	0.284

*Significant at $p < 0.05$

ASD compared to NT ($p < 0.001$). Pb on the other hand was significantly higher in ASD than in NT ($p < 0.000$). Aside this, mean ± SEM levels of oxidative stress markers in ASD and NT showed that TPP and TAC were reduced ($p < 0.010$; $p < 0.017$) while MDA was higher in ASD compared to NT ($p < 0.000$). Although OSI was higher in ASD, the difference between the two groups was not significant ($p > 0$).

Table 2: Correlation of levels of essential and toxic metals level with oxidative markers in NT

There were no significant correlations among the trace metals (including Pb) and oxidative markers in Neurotypical children. However, expressing concentration of Cu as a ratio of Zn showed that Mg positively correlated with Zn/Cu ratio ($r = 0.589$; $p = 0.002$). Cu had negative correlation with Zn/Cu ratio ($r = -0.785$; $p = 0.000$) while Zn positively correlated with the ratio (0.839; $p = 0.000$).

Table 3 is a summary of correlation analysis between trace metals (including Pb) and oxidative stress markers in ASD children

There was a significant negative correlation between Mg and OSI ($r = -0.438$; $p = 0.029$) and a significant positive correlation between Zn and Zn/Cu ratio ($r = 0.907$; $p = 0.000$).

Table 4: Correlation of Zn/Cu ratio with Oxidative indices in Children with ASD

No significant correlations were observed among markers of oxidative stress including with Zn/Cu ratio in children with ASD except between OSI and TAC ($r = -0.983$, $p = 0.000$) where a highly significant negative correlation was obtained.

Table 5: Correlation of Zn/Cu ratio with Oxidative indices in NT Children

The results of correlation analysis of markers of oxidative stress and Zn/Cu ratio in neurotypical children were

Table 2 Correlation of levels of essential and toxic metals level with oxidative markers in NT

		TPP	TAC	MDA	OSI	Zn/Cu
Mg	r	−0.182	0.058	0.006	−0.092	0.589
	P	0.384	0.784	0.979	0.663	**0.002***
Zn	r	−0.128	0.147	0.260	−0.172	0.839
	p	0.542	0.483	0.209	0.411	**0.000***
Cu	r	0.384	0.202	0.095	−0.151	− 0.785
	p	0.058	0.334	0.653	0.472	**0.000***
Pb	r	−0.109	−0.105	−0.036	0.039	0.048
	p	0.602	0.619	0.866	0.853	0.821

*Significant at $P < 0.05$

Table 3 Correlation of toxic and essential metals levels with oxidative stress markers in ASD

		TPP	TAC	MDA	OSI	Zn/Cu
Mg	r	−0.337	0.291	0.284	−0.438	0.378
	p	0.099	0.158	0.169	**0.029***	0.063
Zn	r	0.055	0.158	0.192	−0.106	0.907
	p	0.793	0.452	0.357	0.613	**0.000***
Cu	r	0.222	−0.077	−0.133	0.170	−0.302
	p	0.287	0.715	0.527	0.417	0.142
Pb	r	−0.195	−0.165	0.303	−0.004	0.238
	p	0.350	0.431	0.140	0.986	0.252

*Significant at $p < 0.05$

also not significant. Like in ASD children, a highly significant negative correlation was obtained between OSI and TAC (r = -0.832, p = 0.000); however, contrary to those of ASD, a highly positive significant correlation was equally obtained between OSI and TPP in Neurotypical children (r = 0.696, p = 0.000).

Discussion

This study was designed to determine the interplay of oxidative stress markers (TAC, TPP, MDA, and OSI) and essential and toxic metals (Mg, Zn, Cu, and Pb) in the pathophysiology of ASD. In this environment, this study was the first to examine oxidative stress markers in children with ASD in Nigeria.

The metabolic function of Mg and Ca has been copiously reported [3, 11, 12]; they are also cofactors in many enzymes in the body including those involved in neurogenesis [13].

In heavy metal toxicity, both Mg and Ca may be displaced by heavy metals like Pb thus changing the molecular configuration of the catalyzing enzyme with its attendant negative consequences. Hence, a reduction in level of these essential metals as seen in this study especially in the central nervous system may initiate a deficit in the neurological functions of the metals. Their deficiency may also elicit replacement in some of the enzymatic processes where they are involved or may exacerbate accumulation of other toxic metals leading to deleterious effect of the latter especially oxidation in sensitive organs like the brain. The observed reduced Mg level in children with ASD in this study may clearly corroborate this. Mg deficiency has been reported to increase NO accumulation and lipid peroxidation and lowers plasma antioxidants levels [6, 14]. Its deficiency has also been reported to increase oxidative stress which was reversed by its supplementation in laboratory

Table 4 Correlation of Zn/Cu ratio with oxidative indices in children with ASD

Correlations in ASD						
		TPP	TAC	MDA	OSI	Zn/Cu
TPP	Pearson Correlation	1	−.083	.041	.225	−.180
	Sig. (2-tailed)		.692	.846	.279	.389
	N	25	25	25	25	25
TAC	Pearson Correlation	−.083	1	.360	**-.983**[a]	−.093
	Sig. (2-tailed)	.692		.077	**.000**	.659
	N	25	25	25	25	25
MDA	Pearson Correlation	.041	.360	1	−.369	−.039
	Sig. (2-tailed)	.846	.077		.069	.852
	N	25	25	25	25	25
OSI	Pearson Correlation	.225	**-.983**[a]	−.369	1	.065
	Sig. (2-tailed)	.279	**.000**	.069		.759
	N	25	25	25	25	25
Zn/Cu	Pearson Correlation	−.180	−.093	−.039	.065	1
	Sig. (2-tailed)	.389	.659	.852	.759	
	N	25	25	25	25	25

[a]Correlation is significant at the 0.01 level (2-tailed)

Table 5 Correlation of Zn/Cu ratio with Oxidative indices in NT children

Correlations IN NT						
		TPP	**TAC**	**MDA**	**OSI**	**Zn/Cu**
TPP	Pearson Correlation	1	−.198	−.067	**.696**[a]	−.031
	Sig. (2-tailed)		.344	.752	**.000**	.883
	N	25	25	25	25	25
TAC	Pearson Correlation	−.198	1	−.136	**−.832**[a]	.216
	Sig. (2-tailed)	.344		.518	**.000**	.300
	N	25	25	25	25	25
MDA	Pearson Correlation	−.067	−.136	1	.040	.241
	Sig. (2-tailed)	.752	.518		.849	.245
	N	25	25	25	25	25
OSI	Pearson Correlation	**.696**[a]	**−.832**[a]	.040	1	−.190
	Sig. (2-tailed)	**.000**	**.000**	.849		.364
	N	25	25	25	25	25
Zn/Cu	Pearson Correlation	−.031	.216	.241	−.190	1
	Sig. (2-tailed)	.883	.300	.245	.364	
	N	25	25	25	25	25

[a]Correlation is significant at the 0.01 level (2-tailed)

animals [15]. Hence, findings in this study were similar to earlier works reporting reduced Mg level in children with ASD as a possible basis of observed lipid peroxidation and increased NO accumulation in this disorder.

Zn is also an essential trace metals involved in catalytic activities of over 300 enzymes and plays a crucial role in protein synthesis, immune system and cell division [16, 17]. Zn deficiency has been reported to predispose individuals to development of neuropsychological changes like emotional imbalance, depression and irritability and cognitive disorders [16]. It has also been reported that reduced Zn level exacerbates Cu toxicity [18]. Because of the very crucial roles of this essential metal in many metabolic activities in the body, downregulation of its level as observed in children with ASD in this study in comparison to what obtained in NT children may be significant in the observed dysfunction and clinical symptoms associated with ASD. The expected role of Zn in protein synthesis and cell division may be easily compromised in these children leading to abnormality in DNA synthesis and its attendant effect on genetic composition especially in the brain. One of the metabolic functions of Zn is its modulatory role on Cu absorption at the intestinal level. The redox potential of Cu is also said to be closely linked with its rate of absorption in the intestine [19]. Aside from its role in redox reactions, Cu is also an important cofactor in many metalloenzymes. These antioxidative functions of Cu as a redox metal are largely dependent on the Zn/Cu combination in metallothionein. This molecule has been implicated as regulator molecules in gene expression, homeostatic control of cellular metabolism of metals, and detoxifying agent against toxic metals.

As observed in this study, there was a downregulation of Zn with the attendant imbalance in the Zn/Cu ratio. This might have precipitated a concurrent reduction in the metallothionein level and a deficit in the function of the latter. The consequence of this may be generation of oxidative stress which the imbalance in Zn/Cu ratio may have induced. The imbalance in the level of the two essential metals has been attributed to pathological changes in the intestinal mucosa in ASD leading to a downregulation of Zn and its consequent impairment of antioxidant defenses and impairment of DNA repair especially in sensitive organs like the brain [16, 20–22]. It has also been previously reported that Zinc induces the intestinal synthesis of a copper-binding protein such as metallothionein. Metallothionein traps copper within intestinal cells and prevents its systemic absorption [23]. Thus, a reduction in the expected Zn level as seen from results of this work may have reduced the synthesis of metallothionein and its implication on the modulatory functions of this Zn/Cu molecule in attenuating toxicity of heavy metals.

In this study, Zn/Cu ratio in NT children was 1:1; although some workers have postulated an inverse relationship in levels of the two metals in NT children [24]; hence, however, the observed reduction in Zn level in children with ASD in this study resulting in a reduction in the ratio may precipitate a reduction in the concentration of metallothionein required for the necessary antioxidative activities of the essential metals. Although

results from previous study by Lakshmi and Geetha [25] who found increased Cu levels in ASD, the contradiction may be due to the presence of intestinal malnutrition and malabsorption of Cu in children with ASD in this study. As it has been previously reported, Zn has been associated with Cu absorption in the intestine; unfortunately, presence of malnutrition and/or malabsorption was not investigated in this study.

Hence, it has been proposed that the plasma Zn/serum Cu ratio may be used as a rapid method of determining the functional state of the metallothionein system [26] and by inference the capability of the system to neutralize the toxic effects of heavy metals.

Lead is an established toxicant affecting functions of both central and peripheral nervous systems [27, 28]. Its various domestic and industrial application in the environment and ability to cross biological barrier to accumulate in the internal organs has largely facilitated its toxicity in the body affecting behavior and cognitive functions. The observed elevated Pb level in this study may have been facilitated by the reduction in Zn with the consequent reduction in metallothionein which physiologically could have prevented the accumulation and toxicity of Pb. Results from this study were similar to those of previous studies that reported increased Pb level in children with ASD [29]. The issue of reduced ability to detoxify and eliminate toxic metals like Pb as reported by other workers [19, 30–32] which is largely a function of the amount of metallothionein formed has also been seen in this study.

It may therefore be stated that children with ASD in this study have suffered the pathological consequences of increased oxidative stress due to a reduction in Zn level and the consequent reduction in metallothionein formation leading to accumulation and toxicity of Pb.

Markers of oxidative stress determined in this study were TAC, TPP, MDA, and OSI. The presence of essential metals like Zn, Cu, and Mg in collaboration with other systemic antioxidants was expected to moderate a balance in levels of oxidants and antioxidants to allow for a conducive milieu for normal metabolic activities. This is especially required in sensitive organs like the brain. A reduction in Zn and Mg levels with a concurrent increase in Pb seen in cases in this study may be a good and veritable basis for accumulation of oxidants overwhelming the other antioxidant pathways with the possible creation of an imbalance in the oxidant/antioxidant pool of the body. The reduced TAC level seen in children with ASD in this study could be traced to increased MDA and reduced TPP levels which collectively would overwhelm the antioxidant pool of the body. TPP is an indication of the peroxidase capability in the membrane which with reference to the neuron will have a deleterious effect on its transmission capability. This effect may be accentuated by an increase in MDA level exacerbating the ROS and by implication the oxidation processes at the neural level. The increased Pb level in cases seen in this study may be a clear indication of the source of the increased MDA and reduced TPP which may explain the abnormal cognitive and speech functions characteristic of ASD. Previous studies have reported alteration in composition of fatty acids, oxidation of lipids and phospholipids of the membrane of cells in children with ASD [32, 33]; all these may have direct effect on the functions of proteins that are involved in signal transduction, an important process required in neural transmission.

Elevation of MDA level is suggestive of damage to the lipid component of the cell which may lead to alteration in membrane lipid metabolism, such as composition of fatty acid content of the cell. The ultimate effect of this is its adverse effect on transduction and transmission processes involving these proteins.

Hence, an imbalance in oxidant/antioxidant system of the body especially in sensitive organs like the brain may lead to structural damage due to the deleterious effects of ROS and the attendant disruption in transduction and transmission of signals across neurons. This finding is similar to previous ones where reduced activity of antioxidant enzymes like glutathione peroxidase and superoxide dismutase as well as plasma glutathione concentration was reported in ASD [34–37].

Conclusion

This study showed that decreased levels of Zn and Mg is a possible etiological basis for accumulation of Pb leading to increase in oxidative stress with a reduction in TAC and the attendant abnormal neurological sequelae associated with children with ASD in this environment.

Limitations

Due to the level of understanding of research benefits in the African setting, it has not been easy recruiting participants for this study; this has thus caused the limited sample size of the study. Lack of funding for the project ostensibly due to the same reason has also affected the scope of works carried out in this project. Also, for cultural reasons, non-invasive methods like nail or hair analysis could not be used in this environment. The use of blood analysis in this project has greatly affected the sample size and by implication some of the statistical tools that could be applied in the analysis of results. The sociodemographic data obtained from participants in the study might have indicated variations in the socioeconomic status of the babies while corrections could not be made for multiple comparisons arising from the above differences.

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Authors' contributions
I O Omotosho, corresponding and principal author, conceptualized and designed the project, he also prepared the manuscript. A O Akinade carried out the analytical procedures and did the statistical analysis. I A Lagunju recruited the participants, provided necessary clinical details for the project, and also reviewed the manuscript. M A Yakubu directed and supervised the analytical procedure in his laboratory and also reviewed the manuscript. All authors have read and approved the manuscript and ensured that this is the case.

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Availability of data and materials
All data in support of this manuscript are attached and available.

Declarations

Ethics approval and consent to participate
Approval was obtained from the UCH/UI joint Ethical Committee (UI/EC/15/0087) and Oyo State Ministry of Health Ethical Board (Informed consent was obtained from each participant.

Consent for publication
All authors agreed on this publication.

Competing interests
The authors declare that they have no competing interests.

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INFORMED CONSENT FORM

UI/UCH EC assigned Number: UI/EC/15/0087
This approval will elapse on: 19/08/2016
Title of the research: **TOXIC METALS AND MICRONUTRIENTS STATUS AS BIOMAKERS OF GENETIC ALTERATION IN NIGERIAN ASD CHILDREN**
Name and affiliation of researcher of applicant: **Dr I.O. Omotosho**, Department of Chemical Pathology, Faculty of Basic Medical Science, College of Medicine, University of Ibadan
Sponsor of research: Self sponsored
Purpose of research: The purpose of the research is to find Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research: 10ml of blood will be required from each of the subject the effect of environmental toxicants on a child's developing nervous system and genome in relation pathogenesis of ASD in this environment.
Costs to the participants of joining the research: Your participation in this research will cost you nothing.
Benefit: The goal of this research is to find out if toxic metal and micronutrients status have effects on a child's developing nervous system and alter genome in relation pathogenesis to ASD in this environment.
Confidentiality: All information collected in this study will be given code numbers and no name will be recorded. This cannot be linked to you in anyway and your name or any identifier will not be used in any publication or reports from this study. As part of my responsibility to conduct this research properly, officials from NAFDAC, NHREC and ethics and similar agencies may have access to these records.
Voluntariness: Your participation in this study is purely voluntary.
Alternative to participation: Your refusal to participate in this study will not affect you in any way.
Due inducement: You will not be paid any fees for participating in this research.
Consequences of participant's decision to withdraw from research and procedure for orderly termination of participation: You can choose to withdraw from the research at any time. Please note that some of the information that has been obtained about you before you chose to withdraw may have been modified or used in reports and publications. These cannot be retrieved anymore. However the researchers promise to comply with your wishes as much as is practicable.

If yes, what is it? AA ( ) AS ( ) AC ( ) SC ( ) SS ( ) CC ( )

Do you have diabetes? Yes ( ) N0 ( )

Do you have diabetes in pregnancy? Yes ( ) No ( )

Do you have hypertension? Yes ( ) No ( )

Do you have hypertension in pregnancy? Yes ( ) No ( )

Were you on any medication during pregnancy? Yes ( ) No ( )

If yes, please specify.....

Thanks for your co-operation.

Modality of providing treatments and action to be taken in case of injury or adverse events: If yo suffer any injury as a result of your participation in this research, you will be treated at the Universit of Ibadan Teaching Hospital and the research will bear the cost of this treatment.

What happens to research participants and communities when the research is over: The researcher will inform you of the outcome of the research through a reviewed journal. During the course of this research, you will be informed about any information that may affect your continued participation or your health.

Statement of person obtaining informed consent:

I have fully explained this research to ---------- and have given sufficient information, including the risks and benefits, to make an informed decision.

DATE---------- SIGNATURE----------

NAME----------

Statement of person giving consent: I have read the description of the research or have had it translated to the language I understand. I have also talked over with the doctor to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks, and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have a copy of this consent form and additional information sheet to keep for myself.

DATE----------

SIGNATURE----------

SUBJECT' CODE----------

WITNESS' SIGNATURE----------

Detailed contact information including contact address, telephone, fax, e-mail, and any other contact information of researcher, institutional HREC and head of the institution: This research has been approved by the Ethics Committee of the University of Ibadan and the Chairman of this Committee can be contacted at Biode Building, Room T10, 2nd Floor, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan. Telephone: 08032397993, E-mail: uiuchirc@yahoo.com. In addition, if you have any question about your participation in this research, you can contact: Dr. I.O. Omotosho on 08023342999.

QUESTIONNAIRE

DEPARTMENT OF CHEMICAL PATHOLOGY, FACULTY OF BASIC MEDICAL SCIENCES, UNIVERSITY OF IBADAN

Good day. Ma This questionnaire is being administered to you to evaluate possible contribution of exposure to toxic metals and micronutrients status as genetic make-up of ASD in children. Please respond honestly to the question below. The confidentiality of your response is guaranteed.

Thank you.

DATE..... SERIAL NO .....

I.D NUMBER.....

INDEX NUMBER.....

SECTION A (SOCIODEMOGRAPHIC CHARACTERISTICS)

Child's age (years)..... Age (months)..... Child's sex .....

Mother's age (years)..... Age (months).....

Mother's age at birth of the child (years).....

Father's age at birth of the child (years).....

State of Origin..... Place of residence..... Nationality.....

No of child's siblings.....

Child's birth order .....

Do you have another child with this condition? Yes ( ) No ( )

How many? .....

Mother's occupation .....

Father's occupation.....

Mother's Educational Status: No formal Education ( ) Primary Six Certificate ( ) Senior School Certificate ( ) Graduate ( ) Postgraduate ( )

Father's Educational Status: No formal Education ( ) Primary Six Certificate ( ) Senior School Certificate ( ) Graduate ( ) Postgraduate ( )

SECTION B (ANTHROPOMETRIC CHARACTERISTICS / DEVELOPMENTAL MILE STONES)

Weight (kg)..... Height (m)..... BMI (kg/m2) .....

At what age did your child achieved these?

Stable neck..... Sitting .....

Crawling ..... Walking.....

Talking .....

Do you notice any unusual behaviour in your child? : Yes ( ) No ( )

If yes, describe .....

At what age did you notice the abnormal behavior in your child? .....

SECTION C (ENVIRONMETAL EXPOSURE)

Location of the house: Tarred road ( ) Untarred road ( )

Location of the house with respect to traffic: Heavy traffic ( ), Moderate Traffic ( ) Little traffic ( )

Is your house near a factory, Yes ( ) No ( )

If yes, what is produced in the factory? .....

Is your house painted? Yes ( ) No ( )

If yes, which part? Inside ( ) Outside ( ) Both ( )

Is the paint peeling? Yes ( ) No ( )

Do you have a smoking household member? Yes ( ) No ( )

Primary source of water: Well water ( ) Piped ( ) bottled ( ) others ( )

Do you use insecticides/pesticides? Yes ( ) No ( )

Average time spent away from home in hours.....

Is your house near a dump site? Yes ( ) No ( )

If yes, since when have you being leaving there? .....

SECTION D (DIETARY HISTORY)

How often does your child consume the following?

1. Vegetables and fruits Daily ( ) Weekly ( ) occasionally ( )
2. Nutritional supplements Daily ( ) Weekly ( ) occasionally ( ) none ( )
3. Sea foods Daily ( ) Weekly ( ) occasionally ( )
4. Non-food items Yes ( ) No ( )

If Yes, please state the substance.....

Does your child enjoy placing objects in mouth? Yes ( ) No ( )

If Yes, please state the object.....

Did you take supplement during pregnancy? Yes ( ) No ( )

If yes, specify.....

SECTION E (HEALTH ISSUES)

Were your pregnancies and deliveries normal? .....

Was the birth premature? Yes ( ) No ( ) Birth weight .....

Did any of your children have any health issue at birth?

If yes, what was the health issue? i)measles ( ) ii) jaundice ( ) unusual fever ( )----------

Was the child given oxygen in the first week? Yes ( ) No ( )

Were you vaccinated during pregnancy? Yes ( ) No ( )

Was your child vaccinated? Yes ( ) No ( )

Did your child react negatively to the vaccine? Yes ( ) No ( )

If yes, specify .....

Do you know your child's haemoglobin (Hb) genotype? Yes ( ) No ( )

If yes, what is it? AA ( ) AS ( ) AC ( ) SC ( ) SS ( ) CC ( )

Is your child on any drug? Yes ( ) No ( )

If yes, please specify the type.....

Were you on any medication during pregnancy? Yes ( ) No ( )

If yes, please specify.....

Thanks for your co-operation.

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INFORMED CONSENT FORM

UI/UCH EC assigned Number: UI/EC/15/0087
This approval will elapse on: 19/08/2016
Title of the research: **TOXIC METALS AND MICRONUTRIENTS STATUS AS BIOMAKERS OF GENETIC ALTERATION IN NIGERIAN ASD CHILDREN**
Name and affiliation of researcher of applicant: **Dr I.O. Omotosho**, Department of Chemical Pathology, Faculty of Basic Medical Science, College of Medicine, University of Ibadan
Sponsor of research: Self sponsored
Purpose of research: The purpose of the research is to find Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research: 10ml of blood will be required from each of the subject the effect of environmental toxicants on a child's developing nervous system and genome in relation pathogenesis of ASD in this environment.
Costs to the participants of joining the research: Your participation in this research will cost you nothing.
Benefit: The goal of this research is to find out if toxic metal and micronutrients status have effects on a child's developing nervous system and alter genome in relation pathogenesis to ASD in this environment.
Confidentiality: All information collected in this study will be given code numbers and no name will be recorded. This cannot be linked to you in anyway and your name or any identifier will not be used in any publication or reports from this study. As part of my responsibility to conduct this research properly, officials from NAFDAC, NHREC and ethics and similar agencies may have access to these records.
Voluntariness: Your participation in this study is purely voluntary.
Alternative to participation: Your refusal to participate in this study will not affect you in any way.
Due inducement: You will not be paid any fees for participating in this research.
Consequences of participant's decision to withdraw from research and procedure for orderly termination of participation: You can choose to withdraw from the research at any time. Please note that some of the information that has been obtained about you before you chose to withdraw may have been modified or used in reports and publications. These cannot be retrieved anymore. However the researchers promise to comply with your wishes as much as is practicable.

Modality of providing treatments and action to be taken in case of injury or adverse events: If you suffer any injury as a result of your participation in this research, you will be treated at the University of Ibadan Teaching Hospital and the research will bear the cost of this treatment.

What happens to research participants and communities when the research is over: The researcher will inform you of the outcome of the research through a reviewed journal. During the course of this research, you will be informed about any information that may affect your continued participation or your health.
Statement of person obtaining informed consent:

I have fully explained this research to ---------- and have given sufficient information, including the risks and benefits, to make an informed decision.

DATE---------- SIGNATURE----------

NAME----------

Statement of person giving consent: I have read the description of the research or have had it translated to the language I understand. I have also talked over with the doctor to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks, and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have a copy of this consent form and additional information sheet to keep for myself.

DATE----------

SIGNATURE----------

SUBJECT' CODE----------

WITNESS' SIGNATURE----------

Detailed contact information including contact address, telephone, fax, e-mail, and any other contact information of researcher, institutional HREC and head of the institution: This research has been approved by the Ethics Committee of the University of Ibadan and the Chairman of this Committee can be contacted at Biode Building, Room T10, 2nd Floor, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan. Telephone: 08032397993, E-mail: uiuchirc@yahoo.com. In addition, if you have any question about your participation in this research, you can contact: Dr. I.O. Omotosho on 08023342999.

QUESTIONNAIRE

DEPARTMENT OF CHEMICAL PATHOLOGY, FACULTY OF BASIC MEDICAL SCIENCES, UNIVERSITY OF IBADAN

Good day. Ma this questionnaire is being administered to you to evaluate possible contribution of exposure to toxic metals and micronutrients status in genetic make-up of ASD in children. Please respond honestly to the question below. The confidentiality of your response is guaranteed.

Thank you.

DATE..... I.D NUMBER..... INDEX NUMBER.....

SECTION A (SOCIODEMOGRAPHIC CHARACTERISTICS)

Pregnancy age ..... Parity .....

Mother's age (years)..... Age (months).....

Your age at birth of first child (years).....

Your husband's age .....

State of Origin.....

Place of residence..... Nationality.....

Occupation .....

Husband's occupation.....

Educational Status: No formal Education ( ) Primary Six Certificate ( ) Senior School Certificate ( ) Graduate ( ) Postgraduate ( )

Husband's educational status: No formal Education ( ) Primary Six Certificate ( ) Senior School Certificate ( ) Graduate ( ) Postgraduate ( )

SECTION B (ANTHROPOMETRIC CHARACTERISTICS / DEVELOPMENTAL MILE STONES)

Mother: Weight (kg)..... Height (m)..... BMI (kg/m2) .....

New born: Weight (kg)..... Height (m)..... Head circumference .....

Means of delivery .....

SECTION C (ENVIRONMETAL EXPOSURE)

Location of the house: Tarred road ( ) Untarred road ( )

Location of the house with respect to traffic: Heavy traffic ( ), Moderate Traffic ( ) Little traffic ( )

Is your house near a factory, Yes ( ) No ( )

If yes, what is produced in the factory? .....

Is your house painted? Yes ( ) No ( )

If yes, which part? Inside ( ) Outside ( ) Both ( )

Is the paint peeling? Yes ( ) No ( )

Do you have a smoking household member? Yes ( ) No ( )

Primary source of water: Well water ( ) Piped ( ) well ( ) others ( )

Do you use insecticides/pesticides? Yes ( ) No ( )

Average time spent away from home in hours.....

Is your house near a dump site? Yes ( ) No ( )

If yes, since when have you being leaving there? .....

SECTION D (DIETARY HISTORY)

How often do you consume the following?

1. Vegetables and fruits Daily ( ) Weekly ( ) occasionally ( )
2. Nutritional supplements Daily ( ) Weekly ( ) occasionally ( ) none ( )
3. Sea foods Daily ( ) Weekly ( ) occasionally ( )
4. Non-food items Yes ( ) No ( )

Did you take supplement during pregnancy? Yes ( ) No ( )

If yes, specify.....

SECTION E (HEALTH ISSUES)

Were you vaccinated during pregnancy? Yes ( ) No ( )

Do you react negatively to the vaccine? Yes ( ) No ( )

If yes, specify .....

Do you know your haemoglobin (Hb) genotype? Yes ( ) No ( )

If yes, what is it? AA ( ) AS ( ) AC ( ) SC ( ) SS ( ) CC ( )

Do you have diabetes? Yes ( ) N0 ( )

Do you have diabetes in pregnancy? Yes ( ) No ( )

Do you have hypertension? Yes ( ) No ( )

Do you have hypertension in pregnancy? Yes ( ) No ( )

Were you on any medication during pregnancy? Yes ( ) No ( )

If yes, please specify.....

Thanks for your co-operation.