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Evaluation of Some Biochemical Markers in Child Autistic Spectrum Disorder

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Dedication

- ❖ To my dear country
- ❖ To my father, may God have mercy on him and grant him a spacious paradise, who was my only support after God Almighty.
- ❖ To my mother, I cannot find words to express my thanks to her for what she offered me.
- ❖ To my honorable family and my dear brothers and sisters for their to support me on this long scientific journey.
- ❖ To the children with autism spectrum, especially the child through whom our thoughts came to design this study, wishing him, with God's help, to cross this stage.
- ❖ I am grateful to all of you.

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Summary

Autism is a complex condition defined by many causes one of them being excessive concentrations of necessary and harmful chemicals in children. Thus, this study examined some potential biochemical markers in serum specimens from children with autism spectrum disorder (ASD), such as sphingomyelin, plasminogen, docosahexaenoic acid, homocysteine, folate, vitamin B_{12}, and trace elements specifically zinc (Zn), aluminum (Al), and selenium (Se). The study also looked for the links between biochemical markers levels and autistic severity. This study included 100 participants, 10 of them were excluded and the number became 42 kids with autism spectrum disorder (Gilliam's Scale was used for severity) and 48 healthy kids with age and gender-matched with those of autistic children. According to the severity of autism, children were classified into two sub-groups, medium and under medium case. Graphite furnace atomic absorption spectrophotometry was used for serum trace element analysis while the enzyme-linked immunosorbent assay (ELISA) technique was used for other biomarkers. The results of the study found significant increase in levels of sphingomyelin (OR:4.691, CI:1.289~17.068, p=0.014), plasminogen (OR:7.5, CI:1.844~30.509, p=0.001), and docosahexaenoic acid (OR:5.156, CI:1.412~18.831, p=0.001) when compared medium cases for severity ASD with a control group. In contrast, under medium cases for severity, ASD showed a significant decrease in levels of sphingomyelin (OR:10.33, CI: 2.59-41.256, p=0.001), plasminogen (OR: 0.5, CI: 0.169~0.560, p=0.05), and docosahexaenoic acid(OR: 4.55, CI: 1.30-15.96, p=0.003). The results also revealed notable elevations in homocysteine levels (OR: 2.00, CI: 0.792-5.05, p=0.02) and significant reductions in folate and vitamin B_{12} concentrations [(OR:4.66, CI: 2.615~8.327, p<0.001), (OR: 9.25, CI: 3.66-23.339, p <0.001)] respectively when comparing children with ASD with control subjects, for trace elements found decreases in selenium and zinc concentration (OR: 5.25, CI:

1.96~14.08, $p<0.001$), (OR: 5.25, CI: 1.96~14.08, $p<0.001$), (OR: 3.75, CI: 1.44~9.76, $p=0.02$) and increases in aluminum level (OR: 7.68, CI: 2.64~ 22.34, $p<0.001$) in children with ASD compared to the control group. The area under the curve (AUC) values for most parameters are considered excellent, AUC for sphingomyelin, plasminogen, and docosahexaenoic acid is more than 0.7 in medium and under medium severity ASD cases, while AUC for folate, vitamin B_{12}, Se, and Al ranged from 0.85 to 0.95. These values correspond to high levels of sensitivity and specificity for these parameters. Results indicate a strong positive correlation between ASD and their levels of homocysteine, selenium (Se), and zinc (Zn) (β: 0.43 CI: 0.004~0.083 $p=0.03$), (β: 0.48 CI: 0.280~0.679, $p<0.001$) and (β: 0.31 CI: 0.10~0.52, $p=0.005$) respectively. Besides, the results they were revealed that there is a negative relation between ASD and levels of vitamin B_{12}, folate, and Aluminum (Al) (β: -0.993 CI: -1.418~ -0.568 $p<0.001$), (β: -0.903 CI: -1.335~ -0.471 $p<0.001$), and (β: 0.83 CI: 0.71~0.95 $p<0.001$) when comparing children with autism spectrum disorder (ASD) to a control group. The biochemical markers in this study considered risk factors in autistic children and also suggest using them to diagnose autism spectrum disorder in children specifically markers that have a high sensitivity degree after further study.

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List of Abbreviations

AA	Asperger Assessment
ADI-R	The Autism Diagnostic Interview-Revised
ALA	Alinolenic acid
AQ	Autism Spectrum Quotient
AS	Asperger syndrome
ASD	Autism spectrum disorder
ASDI	The Asperger Syndrome Diagnostic Interview
CG142	clinical guideline
DHA	Docosahexaenoic acid
DSM-5	Diagnostic and Statistical Manual
EPA	Eicosapentaenoic acid
EQ	Autism Spectrum Empathy Quotient
FMR1	fragile X messenger ribonucleoprotein 1
FXS	Fragile X Syndrome
GARS	Gilliam Autism Rating Scale
NICE	National Institute for Health and Care Excellence
PUFAs	polyunsaturated fatty acids
SM	Sphingomyelin
ToM	Theory of Mind (ToM)

Chapter One

Introduction

and

Literatures Review

1.1. Introduction

Autism spectrum disorder (ASD) refers to a collection of diverse neurodevelopmental problems that impact social communication and interaction. However, there is currently a lack of effective medical treatments for the primary symptoms of this disorder [1]. The spectrum of symptoms and the degree of severity of ASD exhibit significant variation across children. The clinical presentation of ASD is contingent upon factors such as the child's age, cognitive and language capabilities, and any accompanying diseases. ASD is defined by deficits in two main domains in the most recent version of the Diagnostic and Statistical Manual (DSM-5). Firstly, there are issues in social communication and engagement, such as socio-emotional reciprocity, using nonverbal methods during social encounters, and forming and maintaining relationships. Second, there are restricted, repetitive, and stereotyped behavioral patterns, such as unusual repetitive movements or behaviors, restricted interests, insistence on sameness, rigid adherence to routines, and sensory challenges that range from seeking to avoiding certain sensory stimuli [2,3]. Nonetheless, the various disruptions in behavior, cognition, and emotions observed in individuals with ASD can be attributed to a significant prevalence of concurrent mental health and medical conditions, such as attention-deficit/hyperactivity disorder ADHD, anxiety, depression, phobias, intellectual disability, speech and language impairments, restrictive and avoidant food intake, sleep disturbances, sensory processing difficulties, and genetic disorders. This frequently exacerbates the complexity and difficulty in identifying, diagnosing, and clinically managing ASD [4,5]. In recent years, there has been a notable increase in the prevalence of ASD in children of several countries [6]. Surprisingly, the global prevalence of ASD is estimated to be 0.76%, implying that approximately one in every 132

children is affected [7]. Autism is more common in children than many other inherited diseases combined, emphasizing its importance as a severe public health issue [8]. In 1952, the Statistical Manual of Mental Diseases (DSM) only had a form for cases of schizophrenia, but it has subsequently been modified to include a larger variety of mental diseases. The greater knowledge and evolution of DSM criteria and diagnostic tools may have contributed to the large increase in new autism diagnoses [9].

The most critical timeframe for brain development is between 0-2 years and that is measured by brain weight. Nevertheless, specific regions of the brain do not reach full maturation by the age of two. Subsequently, brain development persists and progresses for the entirety of infancy and adolescence [10]. Autism spectrum disease is correlated with elevated levels of tension, worry, and social isolation within the relatives of patients [11]. Additionally, ASD places significant economic stress on both society and the families of those affected by the condition [12].

1.2. Definition of Autism

According to the National Autistic Society, autism is a persistent, developmental impairment that impacts an individual's capacity to interact and form relationships with others, as well as their perception of the surrounding environment. The current global prevalence rates of children with any kind of ASD range from 1% to 2% [13]. In Iraq, the estimated prevalence rate is 89.40 per 10,000 individuals [14]. Individuals diagnosed with autism, even if they do not have an intellectual impairment, may struggle with adult psychosocial functioning, as evidenced by measures such as independent living and gainful employment, according to the (DSM-5) [15].

The understanding of results from previous studies on autism spectrum disorder is hindered by various factors. These include the absence of a thorough evaluation of risk factors during pregnancy that are confirmed by maternal biomarkers, limited identification of cases followed until the age when ASD is likely to develop, data collected on physical or developmental measures at only one or two time points, often after the diagnosis of ASD has already been made, reliance on historical controls that may introduce bias, and small to moderate sample sizes that lack sufficient statistical power to identify significant associations.

1.2.1. History of Autism

Since Rutter's groundbreaking study in 1970, the majority of longitudinal studies have found that a small portion of their participants no longer meet the criteria for ASD during follow-up. However, it has been commonly assumed, either explicitly or implicitly, that this outcome is unusual or indicates an initial misdiagnosis [16]. In 1987, Lovaas first brought up the concept of "recovery" or "best outcome". He found that 47% of the individuals in the study achieved average cognitive functioning scores after undergoing an early and comprehensive behavioral intervention program for ASD [17]. In 1911, Swiss

psychiatrist Eugene Bleuler first employed the word 'autism' to describe a condition characterized by social isolation [18]. In 1943, an American psychiatrist introduced the autism concept after a study done on a cohort consisting of 11 youngsters, comprising 8 males and 3 females, who exhibited intellectual impairments and displayed a strong inclination for solitude from a very young age [19]. In the 1960s, Kanner used the term "infantile autism" which gained recognition as a diagnostic for a very uncommon disease largely observed in children with intellectual disabilities. Moreover, in 1944, a Paediatrician from Australia described in detail the characteristics of four male children. These children did not have any intellectual impairment, but they faced difficulties in social communication [20].

1.2.2. Prevalence

An estimated 1-2% of children globally are thought to be affected with ASD [14]. The reported rate for various countries varies based on the accessible research. Although there are reports on the presence of ASD in less-developed nations, the exact incidence rate in many low- and middle-income countries is still unknown. Most studies on this topic have been undertaken in high- and middle-income countries [21]. There is a scarcity of research dedicated to ASD in Iraq. According to the World Population Review website, the anticipated rate of new cases per 10,000 children is 89.40 [14]. In 2022, Nader Salari *et al*. did a meta-analysis that encompassed 74 studies using a combined sample size of 30,212,757 participants. The results indicated that the worldwide occurrence of ASD was 0.6%. The subgroup analysis indicated that the prevalence of ASD in America, Asia, Europe, Australia, and Africa was 0.4%.

1.2.3. Causes of Autism Spectrum Disorder

1.2.3.1. Inheritable Causes of Autism

Hereditary forms of autism are relatively uncommon. One instance is the presence of an X chromosomal defect called Fragile X Syndrome (FXS), which

is a prevalent hereditary factor in intellectual difficulties and makes up 10-20% of single-gene neurodevelopmental disorders. Approximately 30% of children who have been diagnosed with Fragile X Syndrome (FXS) meet the criteria for a provisional diagnosis of ASD [22]. Fragile X syndrome is mostly attributed to the dynamic mutation of a trinucleotide repeat sequence (CGG)n expansion in the fragile X messenger ribonucleoprotein 1 (FMR1) gene. This mutation leads to the absence or reduced expression of fragile X messenger ribonucleoprotein (FMRP) [23]. Fragile X Syndrome is linked to the FMR1 gene and results in the occurrence of several repetitions of the base pairs. This leads to excessive activation of the glutamate receptor in the brain [24]. Rett Syndrome is another genetic disorder associated with the X chromosome. An exceptional characteristic of this illness is the compulsive hand-washing gestures. They have an ungraceful walking pattern and experience a delay in their physical and mental development, with the condition gradually deteriorating over time [25]. Phelan-McDermid Syndrome, commonly referred to as the Shank3 gene, is inherited in an autosomal dominant manner and is linked to autism [26]. Researchers examined mice with genetic deficiencies in the cerebellum and observed reduced neural activity, atypical social interactions, and repetitive behaviors [27].

1.2.3.2. Pharmaceuticals

Attempts to comprehend the heightened occurrence of autism spectrum conditions have prompted speculation over the potential role of diverse medications administered during pregnancy. There is a historical basis to argue for a correlation between autism and thalidomide, a powerful sedative that was administered to pregnant women in the early 1960s to alleviate nausea [28]. A study conducted on a sample of 100 adult Swedish patients, whose mothers had been exposed to thalidomide during pregnancy, revealed that a minimum of four individuals had distinct autistic traits [29]. This finding provided initial

evidence that the consumption of a medication during pregnancy could significantly elevate the likelihood of developing autism. Recently, there have been concerns over the use of valproic acid with serotonin reuptake inhibitors [30].

Valproic acid, a medicine that has been approved since the early 1960s, is mostly used for the treatment of epilepsy and seizure control. However, it is also utilized for various conditions such as migraine headaches and bipolar disorder. Valproic acid is a teratogen in both animal and human epidemiological investigations, which has prompted concerns. The most extensive epidemiological study conducted thus far monitored a total of 415 infants, out of which 201 of whom were born to mothers who took antiepileptic medication during their pregnancies. The prevalence of neurodevelopmental disorders, particularly autism, was significantly higher in the offspring of the treated mothers, with around 7.5 percent affected, compared to only 1.9 percent in the non-epileptic women [31].

1.2.3.3. Environmental Toxicants

In addition to viral and bacterial infections, as well as medically prescribed medications, researchers have initiated investigations into environmental toxicants. These encompass several sources of pollution, including emissions from vehicles, tobacco smoke, as well as the presence of toxic substances such as heavy metals and pesticides. Studies have indicated that living in proximity to a freeway or agricultural region during pregnancy can result in slight elevations in the incidence of autism [32,33]. The study of autism environmental epidemiology is currently in its early stages, and methods that fully construct a prenatal "exposome" (i.e., all environmental elements that impact a fetus during pregnancy) are still being developed. Considering the low probability of genetic variables explaining all cases of autism, identifying and understanding environmental causes, some of which can be prevented or

reduced, may have a more significant practical effect than the extensively financed genetic research.

1.2.3.4. Postnatal Factors

Autism, being a neurological illness, is characterized by abnormal brain function. Consequently, the damage to the brain may arise postnatally. Presently, there exists scant evidence to support this claim. An issue of historical significance revolved around the possibility that vaccines, specifically the measles, mumps, and rubella (MMR) vaccine, given to children around the age of one, could potentially cause a healthy child to develop autism. The dread was intensified by instances of regressive beginnings, where a kid first appears to be developing normally for the first year or so, but subsequently has a decline in social and language abilities, ultimately regressing into a classical autistic diagnosis. Nevertheless, a particular investigation revealed that brain alterations associated with the regressive form of autism can be observed in toddlers as early as four to six months, preceding any noticeable changes in behavior [34]. Moreover, multiple comprehensive epidemiological studies have conclusively demonstrated that there is no correlation between the administration of the MMR vaccine and the likelihood of developing ASD. This finding is consistent with the conclusion obtained by the US National Academy of Sciences in their large study done in 2011[35].

1.2.4. Psychological Theories

In 1985, Baron-Cohen claimed that autism is primarily characterized by a deficiency in the theory of Mind (ToM). The ToM is the cognitive ability to attribute mental conditions, such as convictions, objectives, and desires, to oneself and others. It entails acknowledging that others possess unique views, objectives, intentions, and viewpoints that may vary from one's own. Typically, it is assumed that children who are developing normally begin to develop

(ToM) around the age of three [36]. Deficits in executive functioning have been linked to restricted, repetitive behaviors, activities, or interests [37]. Certain children and young individuals diagnosed with autism exhibit notable talents or exceptional abilities, such as the capacity to memorize through repetition.

Several brain regions in the theory of Mind are shown in Figure1-1, including the prefrontal cortex (PFC), bilateral temporoparietal junction (TPJ), the anterior cingulate cortex (ACC), the inferior frontal gyrus (IFG), and precuneus, have been consistently found to be activated in various mentalizing tasks in healthy individuals [38]. Meta-analyzed imaging studies of ToM contained six different task groups-False belief versus photo, Trait judgments, Strategic games, Social animations, Mind in the eyes, and rational actions. They found the mPFC and bilateral posterior TPJ were activated in all task groups. In the false belief vs. photo stories task group, they found TPJp, IPL, precuneus, posterior cingulate gyrus, mPFC connectivity clusters 3 and 4, ventral parts of the mPFC, anterior cingulate gyrus, right anterior temporal lobe, and adjacent parts of the insula be activated [39].

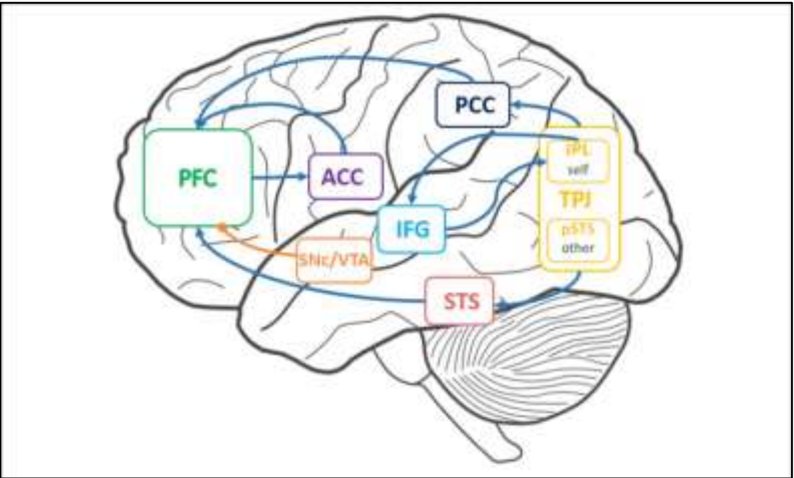

Figure (1-1) The Brain-ToM model(prefrontal cortex PFC, Bilateral temporoparietal junction TPJ, Anterior cingulate cortex ACC, Inferior frontal gyrus IFG, Posterior cingulate cortex PCC, Superior temporal sulcus STS, Inferior parietal lobe IPL) [39]

The second theory is impeded Plasticity is widely known that the brain of autistic children presents functional and morphological dysfunctions. Studies using functional magnetic resonance imaging (fMRI) have already

demonstrated a significant reduction in long-distance connectivity in the brains of ASD individuals [40]. At the microstructural level, disruption of brain development is caused by abnormal regulation of cell division and apoptosis, as well as increased neuronal inflammation [41]. Recently, it has been shown that patterns of both hypo- and hyperconnectivity could be observed in the brains of autistic children [42]. This difference in hypo and hyper-connectivity depends on age-related factors [42]. A study reported that at 3 months old, children who are at high risk for developing autism show increased connectivity compared to low-risk children and that this difference starts to gradually disappear between the ages of 6 and 9 months [43]. There is also evidence suggesting that the autistic brain is characterized by morphological abnormalities such as early overgrowth of several brain structures including the frontal cortex, the amygdala, and the cerebellum [41].

Balanced development of excitatory and inhibitory synapses is essential for the normal function of sensory and cognitive networks in the brain[44]. An imbalance in this development may cause the pathogenesis of several neuropsychiatric disorders summarized in Figure(1-2), including ASD, schizophrenia, and bipolar disorder[44]. In the mature central nervous system, amino butyric acid (GABA)-interneurons send inhibitory synaptic inputs, while glutamatergic neurons send excitatory inputs. Mutations and environmental factors that increase glutamate signaling or decrease GABAergic signaling could lead to an imbalance of excitation and inhibition, and could therefore increase the risk of ASD [45]. There is a significant amount of studies suggesting that individuals with SD have higher than normal glutamate blood levels[46]. GABA, which plays a key role in regulating neuronal excitability, is also found altered in individuals with ASD[47].

Study contact in mice suggests that real-time modulation of the balance between neuronal excitation and inhibition in the mouse medial prefrontal

cortex can rescue social behavior deficits reminiscent of autism phenotypes Figure (1-2).

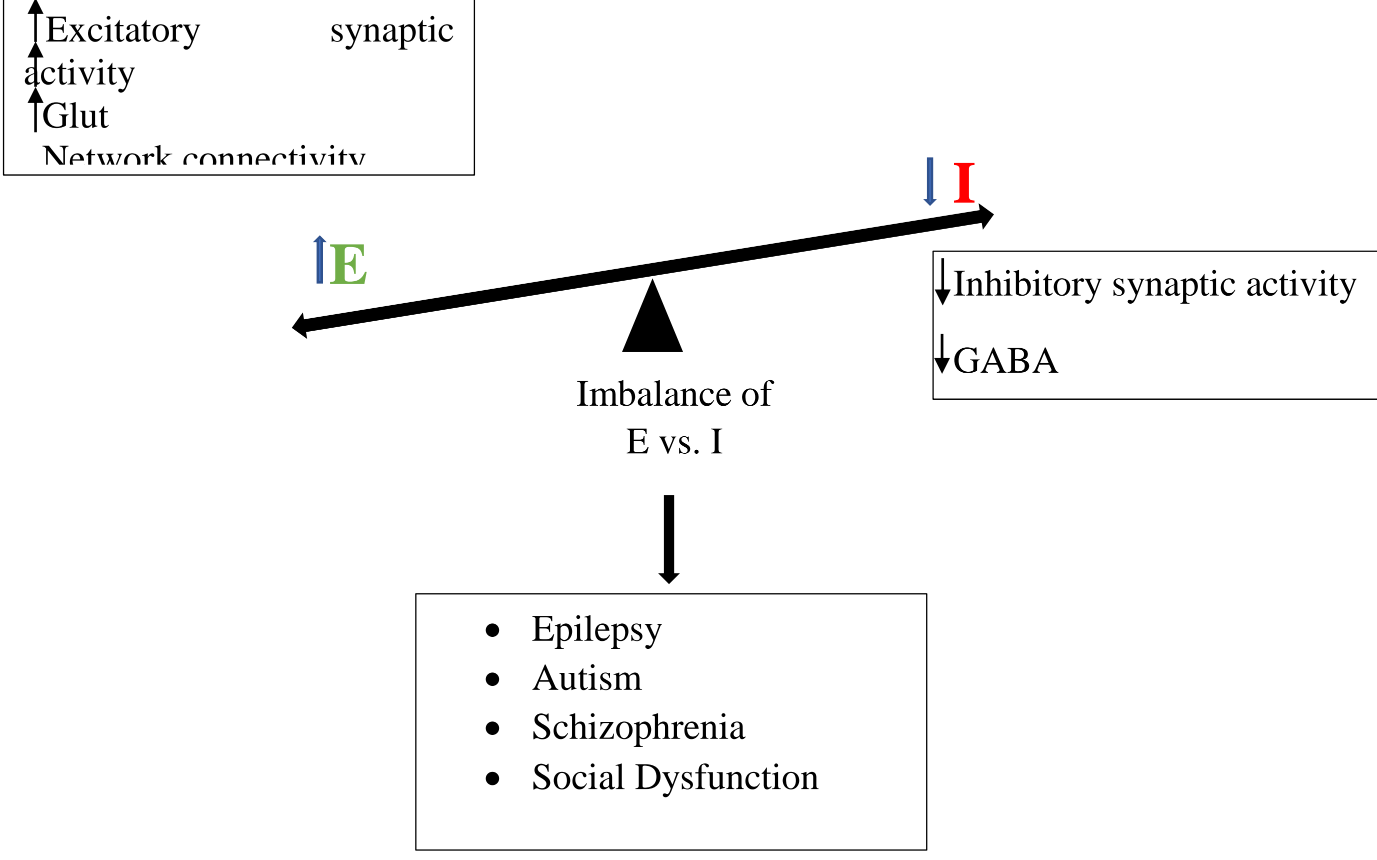

Figure (1-2) The Standard Paradigm for Understanding the Balance between Excitation (E) and Inhibition (I) in Neurodisorders.[48]

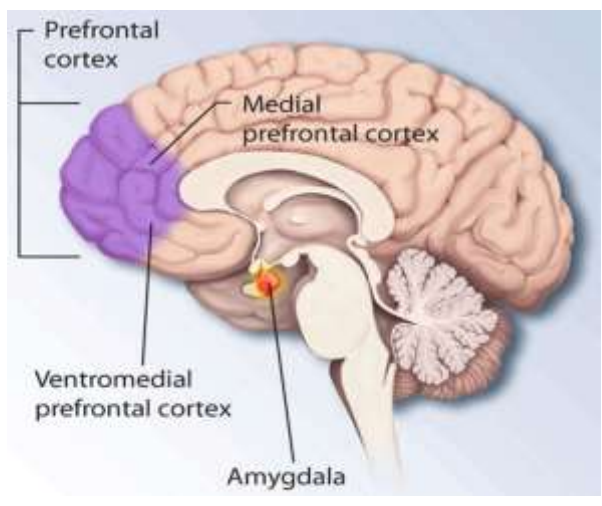

Figure (1-3) The Location of Medial Prefrontal Cortex [49]

1.2.5. Diagnostic of Autism Spectrum Disorder

1.2.5.1. Gilliam Autism Rating Scale (GARS)

Gilliam Autism Rating Scale is an internationally accepted instrument employed to evaluate and diagnose autism as well as other behavioral problems. It depends on reports from parents or teachers on the child's appearance and behavior, and it is a fast assessment to conduct. In addition, there is no need for any substantial training. However, certain studies indicate a propensity to overlook children who would otherwise fulfill the diagnostic criteria for an autistic spectrum disease [50].

The GARS has gained extensive utilization and widespread acceptance. In 1997, a Collaborative Work Group on Autism Spectrum Disorders made a recommendation. The GARS, because of its affordable price and user-friendly nature, has the potential to greatly assist frontline practitioners in the early detection of autism, according to the Quality Standards Subcommittee of the American Academy of Neurology and the Child Neurology Society. GARS is specifically designed for individuals between the ages of 3 and 22 [51]. The GARS questionnaire comprises 56 items that are categorized into four scales: social interaction, communication, and stereotyped behaviors.

1.2.5.2. Asperger Assessment (AA)

The face-to-face interview is divided into four sections (A-D), each of which describes a specific set of autistic characteristics outlined in the Diagnostic and DSM-5. The AA can be divided into four distinct parts. Section A deals with the impairment in social interaction that is qualitative in nature, whereas Section B deals with the patterns of behavior, interests, and activities

that are restrained repetitive, and stereotyped. Section C addresses qualitative deficiencies in both verbal and non-verbal communication, while Section D encompasses limitations in the realm of imagination.

The physician systematically investigates each area to get a variety of examples from both self-reports and reports from others. Additionally, it comprises two self-report questionnaires: The Autism Spectrum Quotient (AQ) and the Empathy Quotient (EQ). The validity of the literature was established using a systematic review, with a sensitivity of 0.92 and a specificity of 1.00 [52].

1.2.5.3. Autism Diagnostic Observation Schedule (ADOS)

Autism Diagnostic Observation Schedule is widely regarded as the most reliable method for accurately identifying autism. It involves a clinician conducting a face-to-face evaluation where they spend time interacting with the kid in an observational manner. Specialized training and participation in reliability coding sessions are necessary. The task may require at least one hour to be fully accomplished. Kupper, Stroth, and his colleagues [53] defined the tool as a standardized semi-structured diagnostic observational regimen. The process entails a structured interview and dynamic exchange between the kid and the clinician. The instrument aims to assess notable social-communicative behaviors, as well as stereotypic and repetitive behavioral characteristics. The ADOS consists of four separate modules specifically tailored for different age groups and levels of linguistic skill. Each module contains a diagnostic method that categorizes individuals as either autistic or non-autistic [53] Few researchers found that the internal consistency and discriminant validity of module 4 of the ADOS-2 were adequate [52]. However, it is important to note that these findings are limited since they solely relied on the COSMIN checklist (COnsensus-based Standards for the selection of health status assessment instruments). Multiple analytical studies

have been carried out on the ADOS-2[18,19]. However, the accuracy and precision seem to differ based on the research and techniques employed to confirm its reliability.

1.2.5.4. The Autism Diagnostic Interview-Revised (ADI-R)

The ADI-R is a systematic informant interview, acquiring a wide range of previous and current information to diagnose autism. Specialized training is necessary and the task typically takes 1-2 hours to finish. Its purpose was to be utilized in tandem with the Autism Diagnostic Observation Schedule 2 (ADOS-2). The ADI-R is designed to systematically and uniformly observe behaviors that are uncommon in individuals without clinical conditions. It primarily assesses three domains of functioning: language and communication, stereotyped behaviors, and reciprocal social interaction. The ADI-R consists of an interview process and five algorithms that are applicable at various ages and endorsed by the National Institute for Health and Care Excellence (NICE) under the reference number NICE No. The clinical guideline CG142, often known as the autism recommendations from 2012, suggests utilizing a formal assessment instrument like ADI-R to assist in diagnosing and assessing individuals with more complicated conditions. However, further validation studies are needed specifically for adults with a person with epilepsy (PWE) [54].

1.2.5.5. The Asperger Syndrome Diagnostic Interview (ASDI)

The Asperger Syndrome Diagnostic Interview (ASDI) is a specialized screening instrument specifically developed to diagnose Asperger syndrome (AS) in persons of all age groups, encompassing both children and adults. It is a concise and organized interview designed to gather information. The assessment comprises a set of twenty open-ended questions that are categorized into six broader domains of autistic traits. To perform the interview, the doctor must possess a thorough understanding of the patient's developmental and clinical

history. In another study, it was claimed that initial findings from a clinical investigation indicate that the inter-rater reliability and test-retest stability are likely to be outstanding, with kappas surpassing 0.90 in both cases [55].

1.3. Critical Perspectives

According to Latif (2016), changes made to the definition of autism have resulted in the broadening of its scope, transforming it from a limited and specific group of behaviors in the 1940s to a more inclusive spectrum in present times [56]. In the film "Hacking" released in 2015, the question is raised as to whether autism should be referred to as a spectrum that is inherent to all humans. On the other hand, Frith's work from 1991 proposes the idea that autism could be seen as a variation of a normal personality [57]. Verhoeff (2012) contends that the notion of autism is inherently linked to the prevalent standards of behavior that are considered either acceptable or unacceptable in modern society [58]. According to a study, autism is characterized as a socially constructed disorder. It is argued that patterns of behavior linked to autism have always been present, but only in recent times have they become a matter of concern due to societal changes [58]. Timimi and McCabe (2016) found that the boundaries of the label have expanded rapidly, although there is limited evidence to support the notion that this growth is driven by novel biological insights into the illness. They perceive the existing definition of autism as a consequence of "ideological shifts" rather than "novel scientific understanding," however acknowledge the difficulties that would arise from ceasing or restricting the application of the term, including the need for a cultural transformation. One publication mentions inquiries about the nature of autism. The author asserts that there is no singular fundamental condition that can be considered the definitive attribute of autism, which explains all the diverse

symptoms exhibited by persons who have been diagnosed with it. The author asserts that despite extensive global research, a definitive genetic or neurological basis for autism has not been uncovered. The writers perceive autism as a broad and flexible notion that encompasses a wide array of traits and symptoms [59].

Hassall (2016) highlights that the present understanding of autism seems to be notably distinct from the description provided by Leo Kanner in the 1940s. This is because the two categories of 'impairment' in the DSM-5 are broader in scope compared to Kanner's original description the initial 18 criteria of autism, as outlined by Kanner and Eisenberg in 1956, described an illness that was considered to be extremely uncommon. This disorder was characterized by a significant absence of emotional connection and the presence of intricate repetitive ritualistic behavior [60].

1.4. Gender Differences

Autism is more prevalent in males than in females. Fombonne (1999, 2003) conducted a comprehensive analysis of the scientific research on autism epidemiology. It was observed that the sex ratios of autism, expressed as the ratio of males to females, varied between 2:1 and 16:1 [61]. Dworzynski *et al*. (2012) discuss the preponderance of males to females, which is 4:1 in the general population. However, this ratio lowers to 10:1 for intellectually able girls and climbs to 2:1 for females with learning challenges [62].

Liability Threshold Model (LTM), Greater Variability Model (GVM) by Wing (1981) [63], and the Brain Differences Model (BDM) by Baron-Cohen (2002) attempt to explain autism's sex differences [64]. Koening and Tsatsanis (2005) summarize the three hypotheses [65]. Schellenberg *et al*. reported in 2006 that boys and girls may have different genes for autism. They suggested

that girls need more "risk genes" to be affected, explaining the larger male-to-female ratios [66].

There is substantial evidence from personal, anecdotal, and clinical accounts that supports the validity of reported sex differences, which should be given due consideration [67]. Possible factors contributing to the increased prevalence of autism diagnosis in males include research studies with a larger representation of boys, the efficacy of existing assessment instruments in identifying the illness in girls, and the divergence in symptoms between males and females.

1.6. Parameters Under Study

1.6.1. Sphingomyelin (SM)

The Sphingomyelin (SM) molecule is composed of two parts: a phosphorylcholine head group connected to a ceramide molecule (Figure 1-4). The latter consists of a sphingosine backbone and a fatty acid (acyl chain). Sphingomyelin (SM) often consists of acyl chains such as 16:0, 18:0, 22:0, 24:0, and 24:1. However, the most prevalent SM species in mammalian tissues is 16:0 [83]

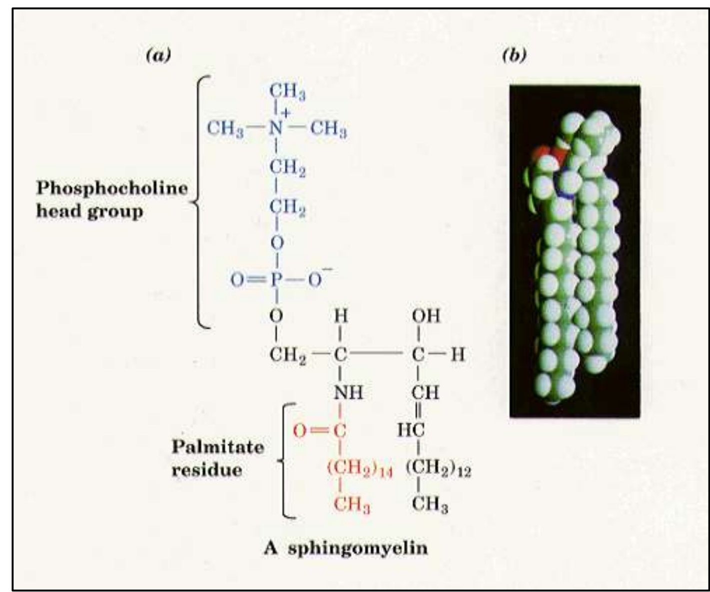

Figure (1-4) Chemical structure of sphingomyelin [83]

Sphingomyelin one of many sphingoid metabolites, is the primary sphingolipid species found in mammalian cells. It is mainly located in the outer layer of the plasma membrane [69]. It serves a vital role in maintaining membrane function and integrity [70]. Sphingomyelin (SM) and cholesterol are key constituents of lipid rafts, which are specialized regions in the plasma membrane that are rich in lipids. These lipid rafts play a crucial role in signal transduction processes [71].

Sphingomyelin (SM) may have a significant role in brain development. Specifically, the substance called SM may have a significant impact from the middle of pregnancy until the end of the first year after birth, during a period when there is a substantial increase in myelin in the central nervous system (CNS) [72]. Center nerve system myelination plays a crucial role in facilitating the effective transmission of nerve impulses along neuronal axons [73]. It is essential for the maturation of brain networks, coordinated information processing, and ultimately cognitive performance in newborns and children [74]. Thus, SM may have a significant impact on cognitive development by its participation in the myelination of the central nervous system, both structurally and functionally [75].

1.6.2. Docosahexaenoic acid (DHA)

Docosahexaenoic acid (DHA) is an omega-3 fatty acid that has a crucial role in the building blocks of the brain, retina, and skin. The abbreviated term for it is 22:6(n-3). DHA can be synthesized metabolically from alpha-linolenic acid (ALA) or it can be obtained from breast milk, fatty fish, or algal oil [76]. The source of DHA in fish and multi-cellular organisms is derived from microalgae. DHA can also be industrially synthesized from microalgae, specifically from *Crypthecodinium cohnii and Schizochytrium sp*. Organisms

that do not consume algal or animal products containing DHA are capable of synthesizing their own DHA from Alpha-linolenic acid. This acid is an omega-3 fatty acid found in plants and is also present in animal products derived from these plants. The presence of DHA in breast milk is crucial for the optimal growth and development of a child [77]. Women exhibit a 15% greater rate of DHA production compared to men [78]. Docosahexaenoic acid is a primary fatty acid component found in the phospholipids of the brain and retina. Figure (1-5)

Figure (1-5) Chemical Structure of DHA [79]

DHA is mostly found in high concentrations as phospholipids, specifically phosphatidylethanolamine, and phosphatidylserine, within neuronal membranes. Phosphatidylserine is the principal acidic phospholipid present in neuronal membranes [80]. Phospholipids are essential components of the brain's solid structure and play a crucial role in neuronal processes. They improve the effectiveness of the synaptic membrane, which is vital for the process of neurotransmission, DHA is also crucial in synaptic signaling [81].

The impact of DHA on neurotransmitters, specifically glutamate enhances synaptic function by increasing the levels of synapsin and glutamate receptor expression in the hippocampus [82]. DHA oversees the actions of glutamate transporters, including Glutamate transporter-1(GLT1), Glutamate

Aspartate Transporter (GLAST), and Excitatory amino acid carrier 1(EAAC1). DHA activates GLT1 and EAAC1 transporters by a process that requires the involvement of extracellular calcium, CaM kinase II, and protein kinase C [83]. Other neurotransmitters inadequate supplementation of DHA can result in a shortage of dopamine in the brain. This shortage can cause dopamine vesicles to be relocated in presynaptic terminals, leading to higher levels of dopamine outside the cells. Additionally, it can also reduce the occurrence of excessive increases in dopamine levels in brain tissues [84]. These findings indicate that alterations in the levels of DHA in the synaptic membrane can impact brain activities, specifically synaptic neurotransmission and plasticity that are regulated by dopamine. DHA also has an impact on the lack of Omega 3 polyunsaturated fatty acids can cause a decrease in serotonin levels, which in turn affects the storage of serotonin and dopamine in vesicles. This can result in the alteration of cerebral receptors in certain regions of the brain, leading to various regulatory processes [84]. Extended deficit in omega-3 polyunsaturated fatty acids leads to changes in serotonin levels in the synapse following activation with fenfluramine. In addition, DHA has an impact on Acetylcholine. The presence of DHA in the phospholipids of the synaptic membrane is enhanced following the activation of choline [85]. Acetylcholine is a neurotransmitter that enhances memory and facilitates learning.

1.6.2.1. Docosahexaenoic acid Composition in the Brain

The brain is the most lipid-rich organ in the body, with lipids accounting for around 60% of its dry weight. Notably, 35-40% of the lipid content in the brain consists of polyunsaturated fatty acids (PUFAs), specifically the long-chain PUFAs Eicosapentaenoic acid (EPA), DHA, and Arachidonic acid (AA) [86]. DHA comprises approximately 15% of the fatty acids found in the frontal cortex of the human brain, indicating a necessary function of this omega-3 fatty acid in brain health [87]. The presence of high amounts of PUFA in the

cerebellum, occipital, and frontal lobes of the brain is considered more evidence for a rise in brain weight during the first few months after birth. This finding also implies that the early developmental phase is particularly responsive to DHA [88]. The central nervous system accumulates DHA during development, which is dependent on the availability of DHA from the circulating plasma. While there is some local production of DHA in the brain, its main source is the diet and the liver, which is the principal site for DHA synthesis. Alinolenic acid (ALA) serves as the precursor for the production of DHA. Mammals are unable to synthesize ALA on their own and must obtain it from their food. Studies conducted on cell cultures indicate that astrocytes, unlike neurons, have the potential to produce DHA [89,90]. This production is regulated by a negative feedback mechanism that depends on the availability of DHA. However, there is a constant baseline synthesis of DHA under all conditions [91]. Cerebromicrovascular endothelial cells, similar to astrocytes, undergo elongation and desaturation of short-chain fatty acids. However, these cells lack the final desaturation step. They work in collaboration with astrocytes to produce DHA [92]. Neurons are primarily responsible for accumulating DHA, which is easily obtained from the discharge of astrocytic membranes in both normal and stimulated situations [93]. However, it is challenging to exhaust DHA from the neuronal membrane in adult mammals [94], even though the activity of Caþþ independent phospholipase A2 (iPLA2), which leads to the release of DHA from astrocytes [95]. *In vitro* investigations have also shown that the brains of □-3 PUFA-deprived rats exhibit a decrease in the activity, protein, and mRNA of the group VIA-independent phospholipase A2 isoform. Conversely, other groups such as cytosolic phospholipase A2 (cPLA2) and secretory phospholipase A2 (sPLA2) show an increase in their levels [96]. Similar to other fatty acids (FAs), DHA in its non-esterified form is mostly delivered to the brain [97].

1.6.2.2. Role of Docosahexaenoic acid on Learning and Memory Function

Population studies indicate the consumption of omega-3 fatty acids is below recommended levels, even in wealthy nations. Additionally, the ratio of omega-6 to omega-3 fatty acids remains significantly higher than the desired level [98,99]. Research conducted on school children has demonstrated significant enhancements in learning and memory [100], reading [101], spelling [100], non-verbal cognitive development [102], processing speed, visual perceptive ability, attention, and executive function [103] following the administration of DHA or a diet supplemented with DHA. Administering DHA supplementation from the 24th week of gestation until delivery, specifically during the critical final trimester, resulted in improved problem-solving skills as assessed by the infant planning test at 9 months of age. However, this supplementation did not have an impact on the development of recognition memory in infants [104]. Additionally, it was found to have a positive effect on the sleep patterns of newborns, which is an important factor in early neurodevelopment [105]. Supplementing with fish oil and cod liver oil is a form of fish oil supplementation that offers a multitude of health advantages. Consuming a significant amount of DHA, which is found in healthy fats, vitamins, and antioxidants, from 18 weeks of pregnancy to 3 months after giving birth leads to a higher score in the Mental Processing Composite of the Kaufman Assessment Battery (K-ABC). These composite measures intelligence in children over 2.5 years old. At 4 years of age, this higher score is beneficial for future mental development [106]. Alternatively, a study indicates that providing lactating mothers with fish oil supplements in the initial four months after giving birth to a full-term baby led to a temporary decrease in language comprehension during the first year. However, this reduction normalized by the age of 2, with no notable enhancement in problem-solving skills [107]. Postnatal supplementation of long-chain polyunsaturated fatty acids (LCPUFA) in milk formulas has been demonstrated to have a minimal or insignificant

positive impact on neurodevelopment in full-term infants. As a result, it is not advised [107]. Nevertheless, the deficient mice did not exhibit any differences in locomotor activity during the open field test or anxiety-related behavior in the elevated plus maze compared to the fully fed controls receiving ω-3 FA. The addition of DHA significantly improved the cognitive function of rats in terms of both reference and working memory [108]. Given the fact that DHA is not easily depleted and has a long half-life of around 2.5 years in the human brain [109], a suitable study was conducted to investigate the depletion of ω-3 PUFA over multiple generations. The findings indicated a reduction in levels of docosahexaenoic acid (DHA) in the frontal cortex and hippocampal regions of the brain, along with compromised cognitive abilities such as alterations in learning, memory, and auditory and olfactory responses [110]. However, the effects were reinstated with the consumption of a DHA-rich diet. Studies on cross-fostering involved newborn pups whose mothers received diets lacking in ω-3 PUFA. These pups were then nursed by mothers who received essential fatty acids during pregnancy and lactation. The results showed a notable increase in cortical and hippocampal DHA content, along with a significant improvement in learning ability as measured by the passive-avoidance procedure [111]. DHA has been demonstrated to accumulate in brain regions linked to cognitive functions, such as the cerebral cortex and hippocampus [112]. The absence of DHA resulted in significant reductions in synapsins and glutamate receptor subunits, leading to a simultaneous decline in long-term potentiation, a cellular process essential for learning and memory. Transgenic Fat-1 mice can naturally synthesize ω-3 fatty acids without relying on ω-3 fatty acid intake from their diet. These mice have a high concentration of ω-3 PUFA in their brain. Transgenic Fat-1 mice have been found to show improved formation of new neurons in the hippocampus, as indicated by increased neuron proliferation and neuritogenesis. This is further supported by an increased density of dendritic spines in the CA1 pyramidal neurons of the hippocampus

[113]. The transgenic Fat-1 mice demonstrated enhanced spatial learning abilities in the Morris water maze, a test that assesses the ability of rodents to navigate using distant cues to find a submerged escape platform. This performance was superior to that of the control littermates [114]. In rats, an imbalanced diet lacking micronutrients during pregnancy has an impact on the amounts of docosahexaenoic acid (DHA) and neurotrophins in the brain after birth. This, in turn, leads to cognitive deficiencies in later stages. There was a significant decrease in the mRNA levels of memory-related proteins, specifically Brain-derived neurotrophic factor, Nerve growth factor, tyrosine kinase B, and CREB. However, this loss can be reversed by taking prenatal supplements of Eicosapentaenoic acid and DHA [115].

1.6.3. Plasminogen

Plasminogen is the inactive form of plasmin. It's a glycoprotein with a molecular weight of 92 kDa. Moreover, it is composed of more than 700 amino acids with only 2% carbohydrate. Plasminogen consists of three domains: five kringles, an N-terminal activation peptide (made of 77 residues), and a protease domain that contains the catalytic triad consisting of histidine, Aspartic acid, and serin [116]. Kringles (80 amino acid residues) are cyclic structures [117]. They are distinguished by a distinctive pattern of disulfide bonding involving the conserved cysteine residues at position 133. The liver is the primary source of plasminogen production, however, there have been reports of extrahepatic synthesis occurring at several locations [118]. The plasminogen's plasma concentration remains generally constant, at around 200 mg per L or 2 µM. However, an elevation is noticed during the acute-phase response [119].

Fantl and Simon's study in the late 1940s discovered that electroconvulsive therapy had the potential to enhance fibrinolytic activity in the bloodstream [120]. This led to future investigations on human brain tissue.

Fantl and Fitzpatrick discovered that brain extracts exhibited fibrinolytic action when mixed with human serum, similar to streptokinase and staphylokinase [121]. The biological origin of the brain-derived plasminogen-activating activity, most likely tissue plasminogen activator (t-PA), was previously identified as neuronal and epithelial [122]. However, many CNS cells display the presence of t-PA [123]. t-PA has been observed to be present in sympathetic nerve terminals, while plasminogen is expressed inside the central nervous system (CNS) [124]. Plasminogen functions are summarized in Figure (1-6).

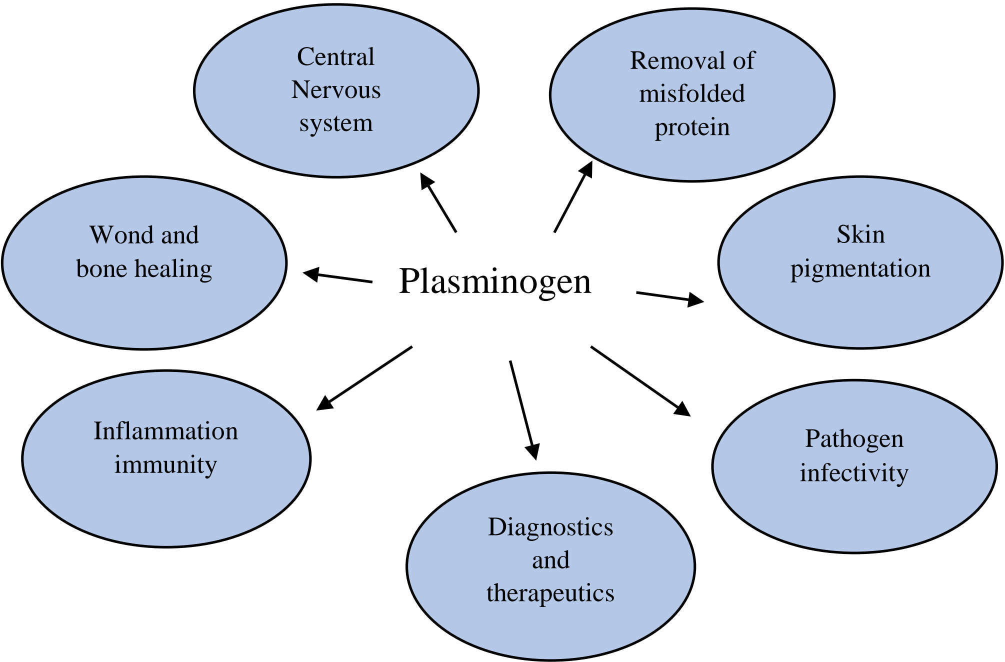

Figure (1-6) Biology function of Plasminogen [123]

In the following years, significant attention was focused on comprehending the function of plasminogen and the plasminogen-activating mechanism in the central nervous system (CNS). Notable functions of this include its role as a neuronal modulator,[125] its ability to promote synaptic plasticity, its involvement in the addictive response [126], and its impact on the permeability of the blood-brain barrier [127]. Some of these effects do not rely on plasmin, but a significant number of them do [128]. The excessive creation of plasmin in the central nervous system (CNS) can have harmful effects in settings when there is an increase in the accumulation of amyloid and fibrin.

This can lead to an excessive generation of plasmin. However, in many central nervous system illnesses, the deposition of fibrinogen plays a crucial role in the neuroinflammatory response. Therefore, the use of medicines specifically targeting fibrin or antifibrinolytic drugs could potentially provide innovative treatments for these conditions [129].

1.6.4. Folate

Folic acid, depicted in Figure 1-7, serves as the fundamental molecule for the broad category of vitamins commonly referred to as folates. Efforts to designate the main molecule based on its chemical structure as pteroylglutamic acid (PGA, PteGlu) have been met with opposition, leading to the continued use of the word folic acid. Naturally occurring folates exhibit one to three structural changes compared to the parent compound [130]. All of the compounds, except 7,8-dihydrofolate (DHF), and 5,6,7,8-tetrahydrofolates (THF), additionally, there is a possibility of substituting a molecule containing one carbon atom at either the N-5, N-10, or 5,10 position. Based on existing evidence, it seems that several THF derivatives found in different investigations are the result of non-enzymatic conversions that occur during the processing of only five specific natural substitutions. These replacements are 5-formimino-THF, 5-methyl-THF, 5,10-methylene-THF, 5,10-methenyl THF, and 10-formyl-THF (Figure 1-8). Furthermore, the quantity of glutamate residues can range from a single residue, as observed in the original molecule, to a maximum of eight residues, which are connected by peptide bonds through the gamma-carboxyl group of the previous glutamate. Distinguishing between various folates can be accomplished using a range of physical, chemical, and microbiological methods. These molecules participate in a sequence of chemical events that transfer one carbon atom. These reactions are responsible for the production of methionine, purines, and thymidine monophosphate, as well as the breakdown of serine, glycine, histidine, formate, and formaldehyde.

Figure (1-7) Chemical Structure of Folate [131]

5,6,7,8-tetrahydrofolates (THF)

7,8-dihydrofolate (DHF)

5-formimino-THF

5-methyl-THF

5,10-methylene-THF

5,10-methenyl THF

10-formyl-THF

Figure (1-5) Chemical structure of 7,8-dihydrofolate (DHF), 5,6,7,8-tetrahydrofolates

Figure (1-8) Chemical Structure of 7,8-dihydrofolate (DHF), are 5,6,7,8-Tetrahydrofolates (THF),5-formimino-THF, 5-methyl-THF, 5,10-methylene-THF 5,10-methenyl THF, and 10-formyl-THF.[132]

Folate is a vital coenzyme that is necessary for transporting groups of single-carbon involved in the production of methionine via biosynthesis. Next, methionine is subsequently transformed into S-adenosylmethionine (SAM) via the transsulfuration process. This procedure facilitates the transfer of a methyl group from methionine to aid in the synthesis of neurotransmitters such as norepinephrine, serotonin, and dopamine. S-adenosylmethionine (SAM) is responsible for inducing epigenetic modifications, which involve reversible alterations to the structure of the genome and the regulation of gene expression, without directly impacting the DNA sequence [133]. In folate metabolism that to across blood-brain barrier been shown in Figure (1-9), the conversion of the biologically inactive folic acid (also known as pteroylmonoglutamate) into biologically active folate derivatives occurs through a single carbon pathway. This process involves a 2-step electron-reduction process catalyzed by dihydrofolate reductase (DHFR). The folic acid molecule is first reduced to dihydrofolate and then further reduced to tetrahydrofolate. Every individual step in the process utilizes one molecule of NADPH, which is obtained from vitamin B_3, and results in the production of one molecule of $NADP^+$ [134]. Serine hydroxy methyltransferase (SHMT) catalyzes the addition of a methyl group consisting of one carbon to tetrahydrofolate, resulting in the formation of 5,10-methylenetetrahydrofolate (5,10-MTHF). This reaction requires co-factors such

as serine and pyridoxine (Vitamin B_6) [135]. Methylenetetrahydrofolate reductase (MTHFR), in the presence of riboflavin (Vitamin B_2), facilitates the transformation of 5,10-MTHF into 5-methyltetrahydrofolate (5-MTHF). This conversion is crucial for the homocysteine methionine S-adenosylmethionine (SAM) cycle, in which cobalamin (Vitamin B_{12}) plays a vital role as a co-factor [136].

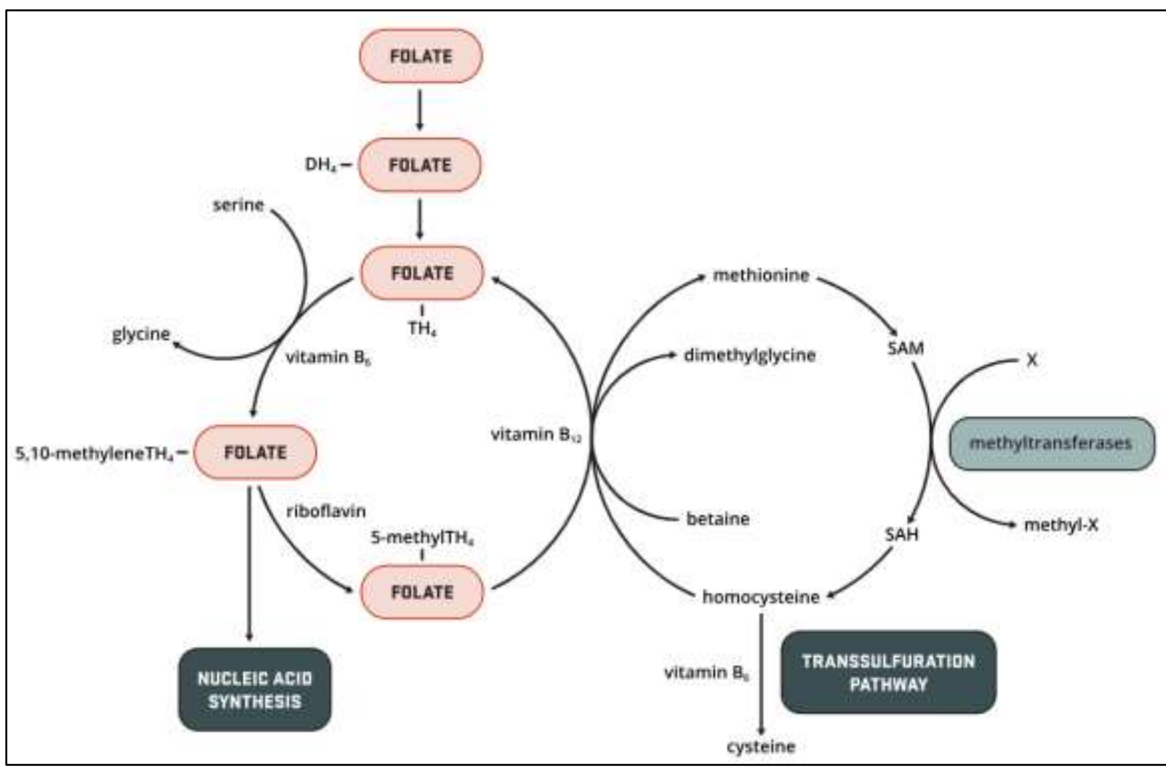

Figure (1-9) Metabolic Steps Required for Different Folate Derivatives to Cross the Blood-Brain Barrier (Tetrahydrofolate TH4-folate, S-adenosylmethionine SAM, S-adenosylhomocysteine SAH) [134].

Any antibody block or prevention for folate receptors that restrict the folate from crossing the blood-brain barrier will reduce and influence the development of the central nervous system(1). Furthermore, in animal models, a decrease in brain folate resulted in homocysteine accumulation as well as

abnormalities in neuronal excitability and maintenance, which contributed to the development of cognitive deficits [137]. Children who have autism spectrum disorder frequently have low dietary intakes, which can lead to severe deficiencies in vitamin B_{12} and folate [138]. This can further increase serum homocysteine levels and make autistic symptoms worse. Folate has also been linked to gastrointestinal issues and/or selective eating [139]. There is therefore the possibility of a disagreement on whether low levels of folate or vitamin B_{12} play a significant role in the etiology of ASD.

1.6.5. Homocysteine

Homocysteine, also known as 2-amino-4-mercaptobutyric acid, is a crucial amino acid structure (Figure 1-10) that is broken down through the remethylation pathways which is explained in (Figure 1-11). This process is dependent upon the presence of cobalamin (B_{12}) and folate vitamins as cofactors. Homocysteine has been classified as a potent excitotoxin, capable of causing harm to neurons and interfering with the production of proteins and neurotransmitters that are crucial for romal brain cell functions. Recent clinical studies have revealed that children with ASDs have higher amounts of homocysteine in their serum and urine than healthy children [140,141]. Surprisingly, ASD children had considerably lower serum cobalamin (B_{12}) and folate levels [142]. Low serum vitamin B_{12} levels in people with ASD imply high levels of oxidative stress and inadequate DNA methylation. These factors are important in the development of ASDs [143]. Cerebral folate deficiency, defined as abnormal folate transport to the embryonic central nervous system, is linked to developmental delays, cognitive deficits, and poor memory function [144]. A study that looked at the relationship between the folate homocysteine metabolic pathway and the development of attention deficit hyperactivity

disorder (ADHD) discovered that variations in genes related to this pathway could influence ADHD development by causing mild hyperhomocysteinemia and vitamin B_{12} deficiency [145]. Previous research has found a link between oppositionality, hyperactivity, and impulsive symptoms in people with Autism Spectrum Disorder (ASD) and Attention-Deficit/Hyperactivity Disorder (ADHD) and vitamin B_{12}, folate, and homocysteine levels [146]. Initial research indicates that vitamin and micronutrient supplementation may potentially decrease emotional instability, aggressiveness, and oppositional behaviors in children diagnosed with ADHD and ASDs [147,148].

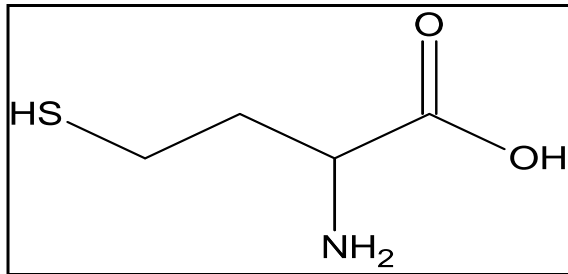

Figure (1-10) Chemical Structure of Homocysteine [149]

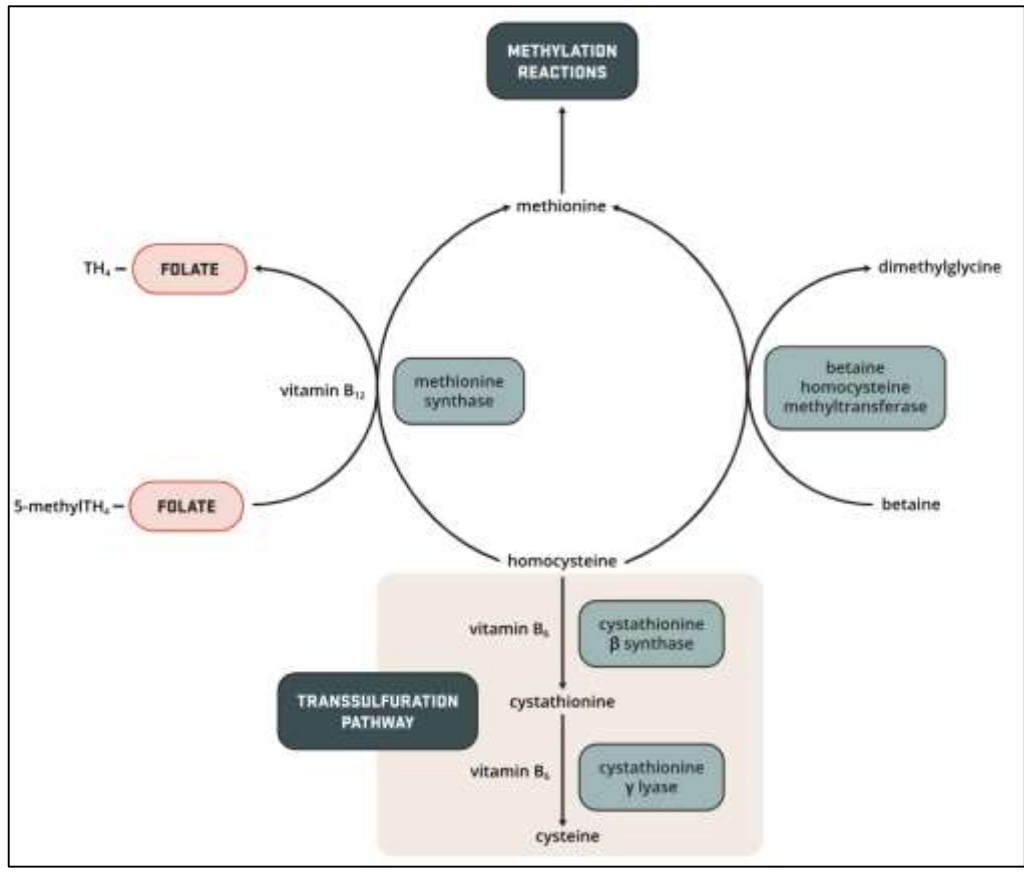

Figure (1-11) Homocysteine Metabolism [150]

1.6.6. Vitamin B_{12}

Vitamin B_{12} is one of the most crucial factors that link to ASD Figure 1-12 illustrates the molecular structure of vitamin B_{12}. The cobalt atom is positioned at the core of a modified tetrapyrrole macrocycle that has undergone ring contraction. It is coordinated by the four nitrogen atoms of the pyrrole rings. The center component of the molecule is known as a corrin ring, which is comparable to but distinct from the ring systems created by tetrapyrroles found in hemes and chlorophylls. A side chain connecting to the macrocycle's ring D connects the corrin ring to a lower nucleotide loop. This lower nucleotide loop contains a one of a-kind base called dimethyl benzimidazole, which also interacts with the cobalt ion. The cobalt ion is coordinated not only by the corrin ring's four pyrrole ring nitrogens but also by the upper (beta) and lower (alpha) ligands. As the higher ligand, the B_{12} molecule is linked to a cyano group, whereas the lower ligand is a nitrogen atom produced from dimethyl benzimidazole [151].

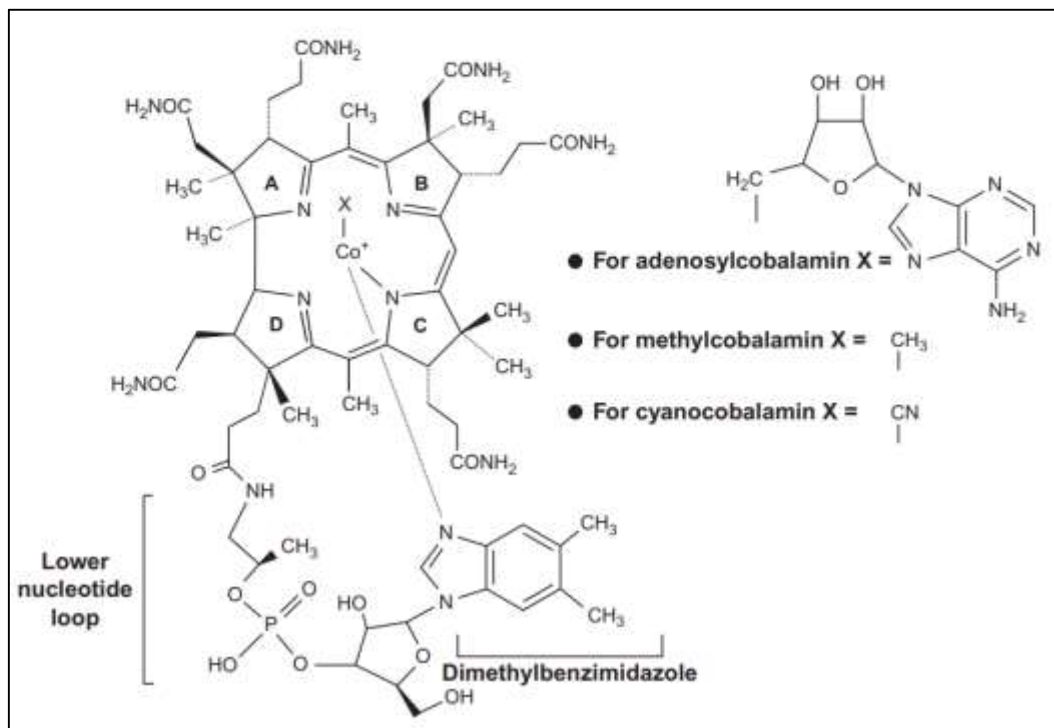

Figure (1-12) Structure of Vitamin B_{12} [151]

Vitamin B_{12} has numerous general benefits such as the synthesis DNA₂, Lipid metabolism, Energy metabolism, Messenger, and Detoxification (Figure 1-13).

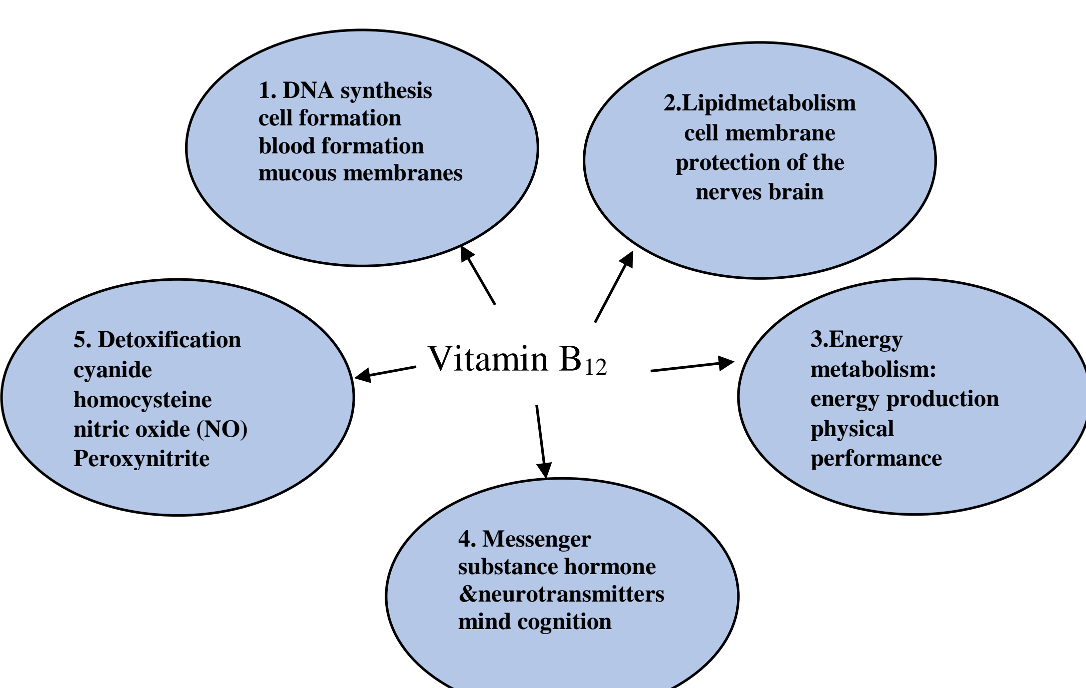

Figure (1-13) The General Benefits of B_{12} [152]

Numerous researches have highlighted the impact of vitamin B_{12} on the motor and cognitive development of children, showing that maintaining a normal level of B_{12} status may also improve the health and development of infants in a broad population [153,154]. An interesting study done in New Delhi involving 538 infants aged 12-18 months where the study assessed their cognitive abilities using Bayley's Mental Development Index, four months after collecting blood samples. The study results showed that the average plasma B_{12} level was measured at 221pmol/L, and it was found that 27% of the infants were deficient in B_{12} (<150pmol/L). In conclusion, the study revealed a direct correlation between plasma B_{12} levels and cognitive performance [155]. Moreover, a deficit in vitamin B_{12} leads to a disruption in the transmission of information inside the brain. In addition, disorganization of myelin sheets of the spinal cord has been associated with B_{12} deficiency. These results in the occurrence of a condition known as "subacute combined degeneration." Furthermore, it is quite probable that it is also the foundation for the neuropathy that is detected in the motor and sensory nerves. One possible explanation for the breakdown of myelin is that the methylation of arginine residues at the 107 position is necessary for myelin protein stability [156].

1.6.7. Trace elements

The huge number of epidemiological data indicates the link between increased environmental contamination, particularly from heavy metals, trichloroethylene, or vinyl chloride, and increased ASD [157]. The build-up of

harmful metals in the organism may seriously interfere with the balance and optimal operation of vital organs like the brain and heart [158]. Heavy metals, such as lead, mercury, and cadmium, are found in high concentrations in the hair of autistic children, even though their blood levels are normal. In motivated study, provided evidence that ASD children have significantly elevated levels of mercury, lead, and cadmium compared to healthy children [159]. Meanwhile, shows an insufficient amount of numerous trace elements in the hair, including zinc, manganese, molybdenum, and selenium, that may be linked to an increased risk of ASD [160]. Various neurotoxic environmental variables have been found through epidemiological investigations, which could potentially lead to neurodevelopmental impairment and ASD. In 2006 and 2012 two studies shed light on xenobiotics as causative agents in the development of (ASD): methylmercury, lead, organophosphorus pesticides, polychlorinated biphenyls, car exhaust fumes, asphaltites, polybrominated diphenyl ethers, polycyclic aromatic hydrocarbons, and per fluorinated compounds. The critical factor in this situation is the combined action and negative consequences of being exposed to multiple xenobiotics simultaneously [161]. Several of evidence confirms that children with autism have elevated levels of dangerous metals, which are linked to the severity of autism symptoms [162]. In a case-control study comparing the levels of metallic elements in the hair of autism children to healthy children, researchers discovered that the levels of molybdenum, lithium, and selenium in autism were higher. [163].

1.6.7.1. Aluminum

The neurotoxic properties of aluminum have been recognized for several decades. Exposure to aluminum mostly arises from the natural environment, where metal is plentiful in water and the earth's crust [164]. However, the main source of aluminum exposure is likely to be food packaging that contains aluminum. Under normal physiological circumstances, the absorption of

aluminum in the intestines is not possible. This is because bioactive metals are only absorbed when they are in the 2+ state. Only in the gas phase following the explosion of aluminized grenades and in interstellar space has the existence of Al^{2+} been observed spontaneously. Aluminum in the stomach's acidic environment is present as the hydrated Al^{3+} ion, namely $[Al\ (H_2O)6]^{3+}$. An increase in pH causes the production of insoluble aluminum hydroxide in the intestines, which is eventually removed in the feces [165]. Both of these processes take place simultaneously. Al^{3+} can only be absorbed by simple diffusion across the weakened intestinal mucosa (due to sickness, inflammation, or intoxication). Another uncommon method is for Al to be absorbed by incontact skin from cosmetic materials [166], as well as by the pulmonary or olfactory epithelium of individuals who come into touch with aluminum in their profession. Aluminum is largely introduced to children through vaccines, where it is used as an adjuvant [167] that bypasses the protective barriers of the gastrointestinal system and skin and is absorbed with 100% efficiency [168]. Aluminum that has been absorbed can indirectly take part in Fenton reactions and efficiently substitute the required bio metals in certain steps of processes [169]. Aluminum forms a complex with transferrin in the bloodstream. Importantly, The Aluminum-transferrin complex exhibits a discernible capacity to inter-brain and cerebrospinal fluid which in turn leads to builds up in the brain [170] and is nearly impossible to eliminate [170]. Approximately 5 milligrams of aluminum from injectable immunizations can reach the infant's body [171]. In light of the confirmation of aluminum neurotoxicity through both mouse experiments and human trials [172]. In 2019, new vaccine techniques proposed that non-toxic zinc compounds (such as phosphate, hydroxide, or sulfate) can be used as adjuvant substitutes [173].

Elevated amounts of aluminum (Al) were identified in the brain tissue of ASD children [173]. Fluorescence microscopy approach was used to detect the level of Aluminum. In detail, Aluminum was detected in non-neuronal and

neuronal cells in different brain loci (gray and white matter, blood vessels). Detecting particular locations of aluminum within brain tissue may aid in determining the role of aluminum in autism spectrum disease. Additional studies on the relationship between aluminum pollution and the prevalence of ASD, on the other hand, appear to be ambiguous [174,175]. The majority of these investigations utilized patients' hair as the primary material for analysis, while blood or urine samples were less frequently employed. Aluminum (Al) in autism has been studied further since pediatric vaccines with aluminum adjuvant are linked to an increase in ASD [176].

1.6.7.2. Zinc

Zinc (Zn) is a vital micronutrient. Moreover, Zn has a high impact on many crucial biological processes [177]. Zinc is a crucial metal that is essential for the normal physiology function and growth of the CNS. Furthermore, zinc is a necessary component of many transcription factors and enzymes. Its principal activities include spatial structural stabilization and functioning as a cofactor in substrate catalysis [177]. Interestingly, Zinc is absorbed in the first part of the small intestine. Nevertheless, the amount of zinc that may be effectively absorbed by the body is limited, with an estimated bioavailability of approximately 20-40%. Divalent metal transporter 1 (DCT1), which is found on the brush border membrane, is the primary protein that mediates zinc absorption [178]. After zinc enters the cell, it will become part of two pathways: it can become a component of secretory vesicles or it can form complexes with metallothioneins by attaching to nitrogen atoms, SH groups, and carboxyl groups of amino acids, particularly cysteine, and histidine [179]. Zinc ions in the plasma are complex with α-2-macroglobulin and albumin [180]. Metallothionein is considered to safeguard cells in the nervous system by effectively sequestering surplus zinc ions, hence preventing their toxicity and associated hazardous outcomes. Zinc serves as a fundamental element in zinc

finger proteins and has an impact on specific genes that are known to be linked to the progression of autism [181]. In many types of congenital metabolic disorders (mitochondrial DNA abnormalities), high levels of reactive oxygen species (ROS) stimulate the production of metallothionein. This, in turn, leads to a reduction in the plasma concentration of zinc due to its interaction with metallothionein. Furthermore, any increase in the production of metallothionein in enterocytes likely contributes to the hindered uptake of Zn in the small intestine. Several researchers propose a correlation between low levels of zinc with ASD [182,183]. Zinc, on the other hand, plays a key part in the immune system, and any deficiencies would result in immunodeficiency. Nevertheless, the available evidence on the impact of zinc on cytokine levels presents inconsistent findings. A high zinc level can influence the production of several cytokines from peripheral mononuclear cells [184]. Individuals with a severe deficit of zinc have been verified to exhibit neuropsychological abnormalities, cognitive dysfunctions, and mental retardation [177]. One study provides evidence that a lack of zinc throughout pregnancy, fetal development, and infancy might lead to abnormalities in the formation of cognitive skills and potentially contribute to the onset of autism [185]. The vast majority of studies on zinc levels in children with ASD show a significant decrease in zinc elements between the ages of 0 and 3 years. In addition, animal studies have shown that immature individuals who are often exposed to a lack of this specific microelement experience impaired cognitive function and working memory due to disrupted development of the neural connection network [186,187]. Nevertheless, this phenomenon experienced a substantial decline after addressing the inadequacies and had no impact on long-term memory [188]. The identified neurological diseases are linked to the metal's unique role in modulating synapse plasticity, regulating excitation, and conducting nerve signals [189].

1.6.7.3. Selenium

Selenium (Se) plays a crucial role in sustaining neurological functions. Se-deficiency causes the brain to prioritize the retention of selenium over other tissues [190], leading to specific protection against deficiency. Selenoprotein P serves as the primary supply of selenium for the brain and selenium distribution inside the brain [191]. The physiological functioning of organisms is significantly impacted by deficiencies, as evidenced by many knockout mouse models [192]. Selenoprotein P is produced by both hepatocytes and astrocytes, hence facilitating the dispersion of selenium in the brain. The brain is extremely vulnerable to oxidative stress [193], which is recognized as a molecular process underlying the progression of neurodegenerative disorders (NDs). Many selenoproteins have antioxidant capabilities that could potentially mitigate the progression of neurodegenerative disorders [194]. An association between reduced levels of selenium in the blood and cognitive impairment has been documented in older individuals [195]. Furthermore, two major neurodegenerative diseases Alzheimer’s disease and Parkinson's disease patients had lower plasma Selenium (Se) and Selenoprotein P levels [196].

In a comparison study between ASD and normal children, the Se levels were very low in ASD children. Specifically, the frequency of selenium insufficiency was evaluated through hair analysis. The prevalence of ASD was markedly elevated in the group of children from Georgia compared to the control group [156]. Research has shown that the levels of selenium (Se) in the hair of children with low-, medium-, and high-functioning autism were respectively 83%, 41%, and 24% lower than the levels observed in the control group. The study found that nail Se levels were substantially lower in individuals with low-, medium-, and high-functioning autism when compared to the control group [197].

In one study, the levels of selenium in red blood cells (RBC) were seen to be reduced by 15% ($p = 0.0006$) in a group of Canadian autism children, in comparison with the control group [198]. Furthermore, there has been a notable ($p < 0.001$) reduction in red blood cell selenium (Se) levels observed in ASD children in comparison to the control group. Besides, there was a correlation between reduced selenium (Se) levels and increased levels of lead (Pb) and mercury (Hg) in ASD children [199]. In the same sample, the levels of selenium in the cerebellum showed only a modest drop in ASD children.

A previous study observed significantly lower levels of plasma selenium (Se) in children with ASD compared to the control group [200].On the other hand, numerous studies have shown that there is no significant association between selenium metabolism and ASD. A study conducted in Arizona examined 55 children with autism and 44 children without autism, all between the ages of 5 and 16. The study found that the levels of selenium (Se) in both whole blood and red blood cells (RBC) were almost identical across the two groups [201]. The meta-analysis results also indicated the absence of a correlation between hair selenium levels and ASD [202]. Furthermore, a recent meta-analysis revealed that there was no significant disparity in the levels of selenium in both hair and red blood cells between children with ASD and those without ASD (control children) [203]. Similarly, out of ten patients diagnosed with (ADHD) and (ASD), only two exhibited a notable decrease in erythrocyte selenium levels [204]. Blaurock-Busch *et al*. (2011) showed that hair selenium levels in autistic children were almost half of those in non-autistic children. However, this difference was not statistically significant due to the large variation in the data [205]. Although numerous researches have shown different indicators and rates of selenium deficiency in ASD children, certain investigations have shown elevated levels of selenium in the hair of autistic children [206].

1.7. Knowledge Gap and Importance of the Study.

Autism is a complex disorder linked to several genetic abnormalities and nutritional variables. Moreover, these aspects have been well-researched in several studies. The association between Autism and these characteristics has not been thoroughly examined in a single study, also clinical research on ASD is little, additionally, increase in the prevalence of ASD in recent years in light of technological taking place development but no accurate clinical methods diagnose it. What was mentioned above are the Knowledge Gaps on this subject, and this study is an important comprehensive attempt to investigate the association between Autism and putative biochemical markers to diagnose ASD.

1.8. The Research Questions

- ❖ Are parameters under study used to diagnose autism spectrum disorder?
- ❖ Is there an association between parameters under study and autism?
- ❖ Are parameters under study considered risk factors in autistic children?

1.10. The Aim and Objectives of the Study

The research aims to find biochemical markers used to prognosis and diagnose autism.

To achieve the aim, the following objectives were followed: -

1. Evaluation of sphingomyelin, plasminogen, and Docosahexaenoic in serum of autistic children
2. Evaluation of homocysteine, folate, and vitamin B_{12} in serum of autistic children
3. Evaluation of trace elements (Se, Al, and Zn) in serum of autistic children
4. Finding the correlation between parameters under study and autism.
5. Finding if there is any association between (sphingomyelin, plasminogen, Docosahexaenoic, homocysteine, folate, and vitamin B_{12}, Se, Al, and Zn) and autism

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Summary of the Study Design

The summary of study design is shown in Figure 2-1.

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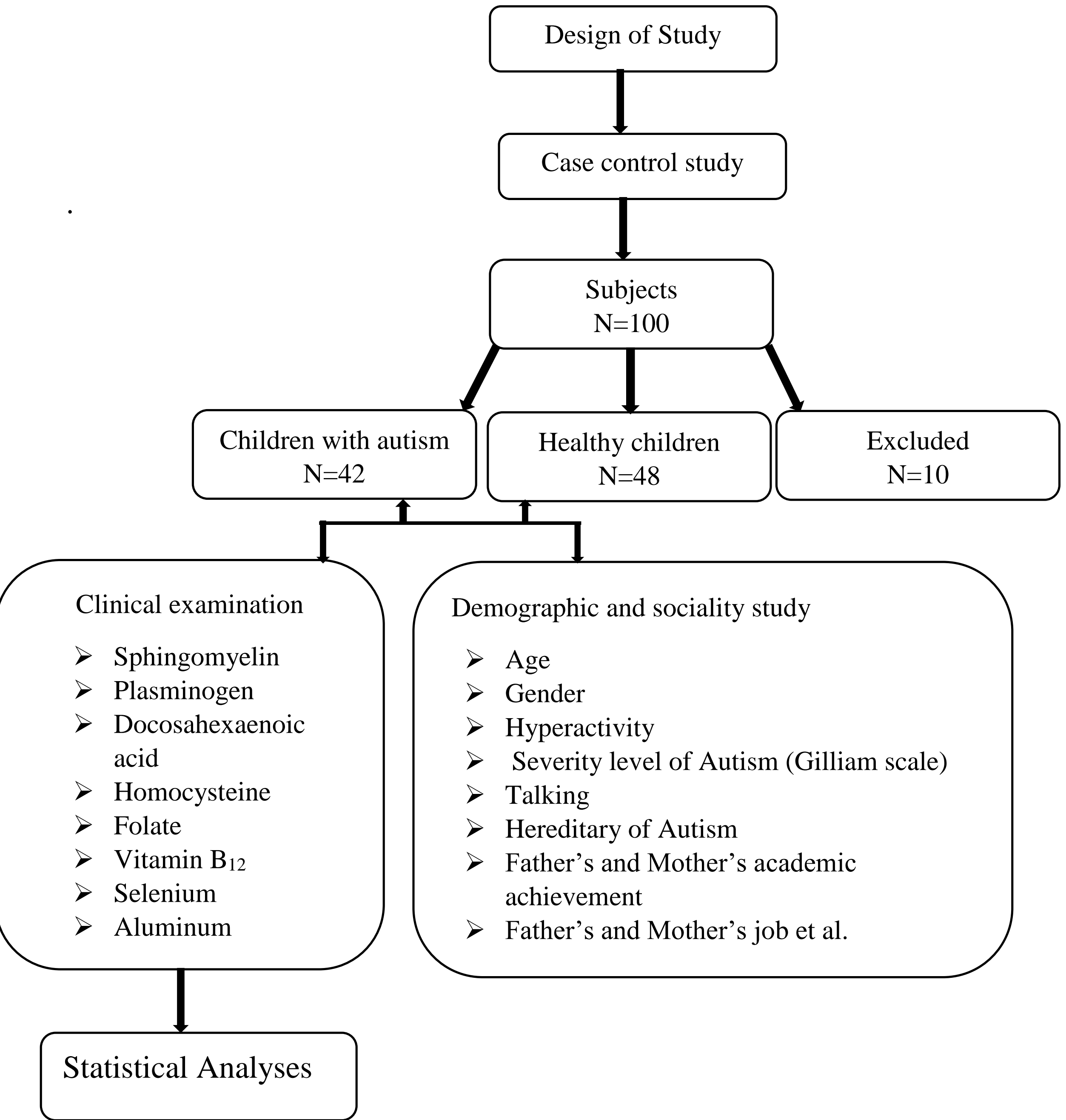

Figure (2-1) Scheme of the Experimental Part

2.2. Subjects and Design of Study

The current study is a case-control study that comprised two groups. The first group consisted of 42 children diagnosed with autism spectrum disorder (ASD) at the Hamaem Al-Salam Center in Iraq/Najaf. The second group which is served as the control group included 48 children, and the period to collect specimens extended from October 2022 to February 2023. The Gillim scale was employed to assess the severity of autism in the children. Ultimately, the age range of participants in both groups was from 3 to 12 years.

2.2.1 Exclusion Criteria

Children with Down syndrome, obsessive-compulsive disorder, mental disorders, or any additional psychiatric or neurological diseases were excluded from this study.

2.2.2. Inclusion Criteria

- Children with an autism spectrum disorder.
- Age (3-12) years.

2.3. Control group.

Forty-eight healthy children were included in this study. They were matched in their sex and age with the ASD group. They were from family, relatives, and friends.

2.4. Data Collection

2.4.1. Questionnaire

A questionnaire was designed to obtain information about ASD children. It contained the name, sex, age, ASD level, hyperactivity, living, talking, and family history of ASD…*etc* (Appendix A).

2.4.2 Examination of the Severity of Autism

The severity of ASD level was determined using the Gilliam scale (Appendix B).

2.5. Approval of the Ethical Committee

The study involved the collection of blood samples from children with autism, following the necessary administrative procedures, including obtaining consent from parents. This process was conducted under the supervision of the Dean of the College of Science and the Head of the Chemistry Department at the University of Kerbala, with oversight provided by the professor overseeing the research. The specimens were obtained from the participants at the central site situated in Najaf, Iraq.

2.6. Materials

2.6.1. Apparatuses and Tools

The apparatus and supplied company are listed in Table 2-1

Table (2-1) Instruments and their Origin Used in this Study

Name	Company	Country
Centrifuge	Hettich	Germany
Micropipette	SHIMADZU	Japan
ELISA washing machine	Stat Fax 2600	USA
Spectrophotometer	SHIMADZU AA-6300)	Japan
Incubator	Stat Fax2200	USA
Pipette tips (Blue & Yellow Tips)	CitoTes	China
Vortex mixer	Lka Genius3	USA
ELISA reader	ChroMate	USA

2.6.2. Diagnostic Kits

The diagnostic kits and their supplier are summarized in Table 2-2

Table (2- 2): Diagnostic Kits and their Supplier Used in this Study

Kit name	Company	Country
Human sphingomyelin ELISA kit	BT LAB	China
Human Plasminogen ELISA kit	BT LAB	China
Human Docosahexaenoic acid ELISA kit	BT LAB	China
Human Homocysteine ELISA kit	BT LAB	China
Human Folate ELISA kit	BT LAB	China
Human Vitamin B_{12} ELISA kit	BT LAB	China

2.7. Methods

2.7.1. Blood Specimens Collection

Venous blood samples were obtained from ASD children and healthy children in volume 5Ml.Specimens were put in a gel-tubes at room temperature and then were allowed to clot, after that the tubes were centrifuged at 3000 xg for 10 minutes. The serum was separated and kept in the Eppendorf tube at a temperature of - 60°C until use.

2.7.2. Determination of Sphingomyelin

- **Principle**

This kit is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) used to determine SM. The plate has been pre-coated with a human SM antibody. Sphingomyelin (SM) present in the sample is added and binds to antibodies coated on the wells. And then, biotinylated Human SM Antibody is added and binds to SM in the sample. Then, Streptavidin-HRP is added and binds to the Biotinylated SM antibody. After incubation, unbound Streptavidin-HRP has washed away during a washing step. A substrate solution is then added and

color develops in proportion to the amount of Human SM. The reaction is terminated by the addition of an acidic stop solution and absorbance is measured at 450 nm.

- **Reagents**

Components	Quantity (96T)
Standard Solution (160mg/dl)	0.5ml x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human SM Antibody	1ml x1

- **Preparation of Standard Solutions:**

A 120µl of standard (160 mg/dL) was reconstituted with 120µL of standard diluent to generate an 80mg/dL standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation before making dilutions. standards were prepared by serially diluting the standard stock solution (80mg/dL) in a ratio 1:2with standard diluent to produce 40mg/dl, 20mg/dL, 10mg/dL, and 5mg/dL solutions (Figure 2-2). Standard diluent serves as the zero standard (0 mg/dL). Table 2-3 shows the dilution of standard solutions.

Table (2- 3) Dilution of Standard Sphingomyelin

80mg/dl	Standard No.5	120µl Original Standard + 120µl Standard Diluent
40mg/dl	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
20mg/dl	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
10mg/dl	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
5mg/dl	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

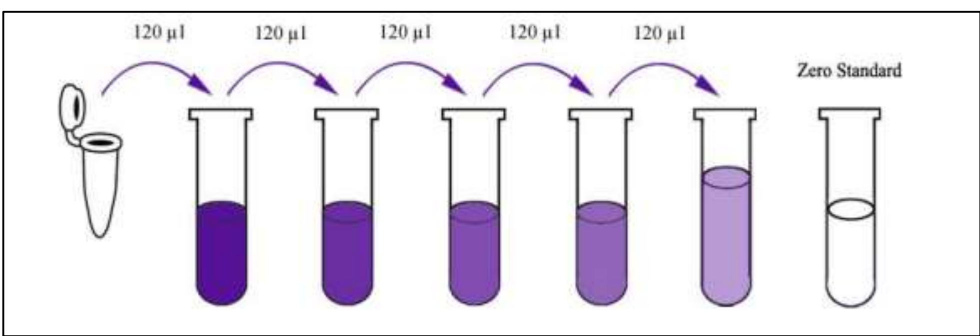

Figure (2-2) Dilution of Sphingomyelin Standard

- **Procedure**:

1. A 50µL of the standard was added to the well with the noted do not add biotinylated antibody to the standard well because the standard solution contains biotinylated antibody
2. A 40µL of the sample was added to sample wells and then 10µL anti-SM antibody was added to sample wells and 50µL streptavidin-HRP was added to sample wells and standard wells (Not blank control well). Well was mixed. The plate was covered with a sealer and incubated for 60 minutes at 37°C.
3. The sealer was removed and washed the plate 5 times with wash buffer. Wells were automated washing, aspirated or decanted was done for each well and washed 5 times with wash buffer. The plate was blotted onto paper towels.

4. A 50µL of substrate solution was added to each well and then a 50µL of substrate solution B was added to each well. The incubated plate was covered with a new sealer for 10 minutes at 37°C in the dark.
5. The stop solution (50µL) was added to each well, and the blue color changed into yellow immediately.
6. Each well's absorbance was immediately determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

- **Calculation**

The concentration of SM is calculated using a standard curve. A standard curve was constructed by plotting the absorbance for each standard on the y-axis against the concentration on the x-axis (Figure 2-3).

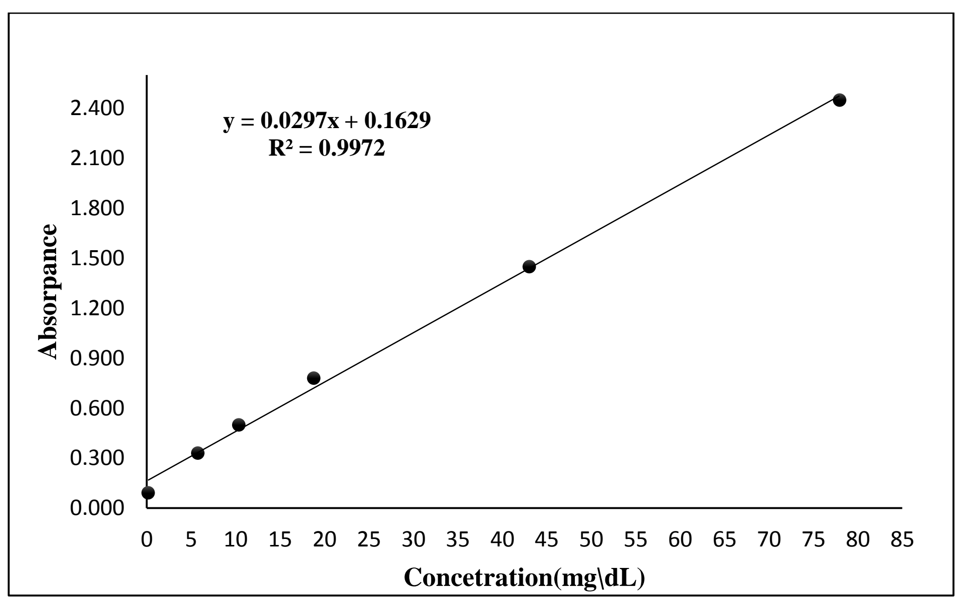

Figure (2-3): Standard Curve of Sphingomyelin

2.7.3. Determination of Plasminogen

- **Principle**

The assay principle of plasminogen is the same as the principle of sphingomyelin. It was mentioned above in the paragraph 2.7.2.

- **Reagents:**

Components	Quantity (96T)
Standard Solution(400ng/ml)	0.5ml x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human PLG Antibody	1ml x1

- **Standard Preparation:**

A120µL of the standard (400ng/mL) was reconstituted with 120µl of standard diluent to generate a 200ng/mL standard stock solution. The standard was allowed to be set for 15 minutes with gentle agitation before making dilutions. Standard points were prepared by serially diluting the standard stock solution (200ng/mL) in a ratio of 1:2 with standard diluent to produce 100ng/mL, 50ng/mL, 25ng/mL and12.5ng/mL solutions (Figure 2-4). Standard diluent serves as the zero standard (0 ng/L). Table 2-4 shows the dilution of standard solutions.

Table (2-4) Dilution of Standard Plasminogen

200ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
100ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
50ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
25ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent

12.5ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

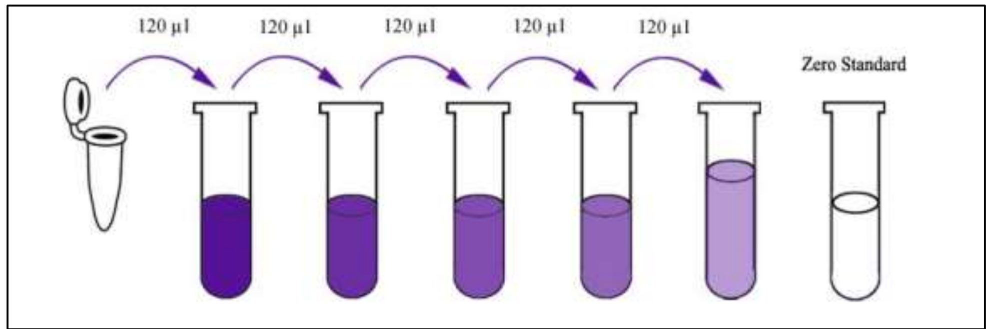

Figure (2-4) Dilution of Standard Plasminogen

- **Procedure**

1. A 50µL of the standard was added to the standard well with a note not to add a biotinylated antibody to the standard well because the standard solution contains a biotinylated antibody.
2. A 40µL of the sample was added to sample wells then added 10µL anti-PLG antibody to sample wells, and then 50µL of streptavidin-HRP was added to sample wells and standard wells (Not blank control well). The wells were mixed and then the plates were covered with a sealer. The plate was incubated for 60 minutes at 37°C.
3. The sealer was removed and washed the plate 5 times with wash buffer. Wells were soaked with 300µL wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirated or decanted were done for each well and washed 5 times with wash buffer.
4. A 50µL of substrate solution was added to each well and then a 50µL of substrate solution B was added to each well. The incubated plate was covered with a new sealer for 10 minutes at 37°C in the dark.
5. The stop solution (50µL) was added to each well, and the blue color changed to yellow immediately.

6. Each well's absorbance was immediately determined using a microplate reader.

- **Calculation**

The plasminogen concentration is calculated using a standard curve. A standard curve was constructed by plotting the absorbance for each standard on the y-axis against the concentration on the x-axis (Figure 2-5).

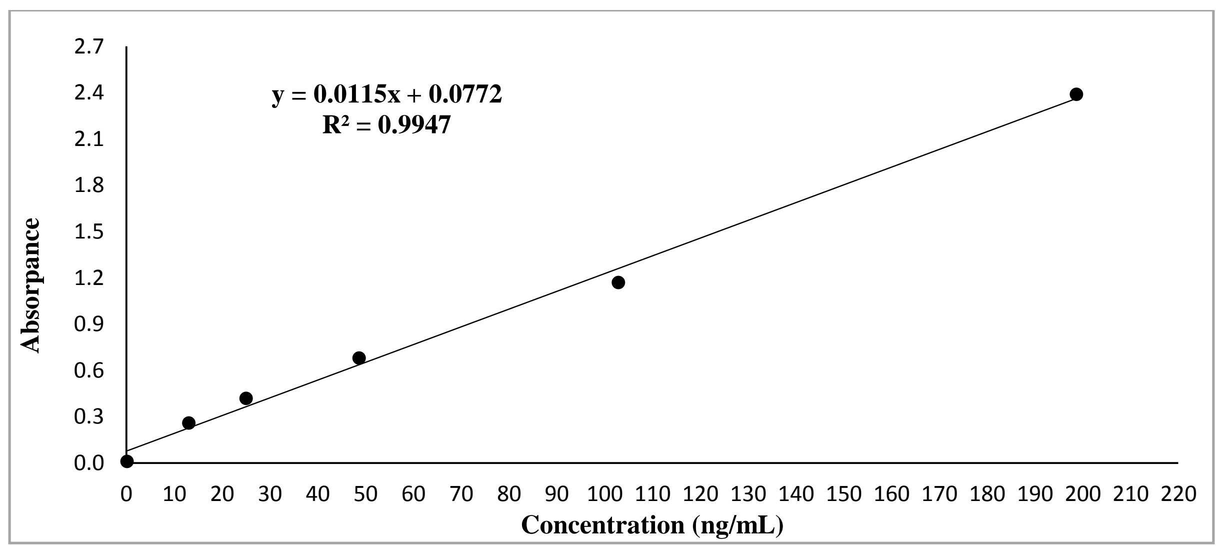

Figure (2-5): Standard Curve of Plasminogen

2.7.4. Determination of Docosahexaenoic Acid

- **Principle**

The assay principle of docosahexaenoic acid same as the principle of sphingomyelin. It was mentioned above in the paragraph **2.7.2.**

- **Reagents**

Components	Quantity (96T)
Standard Solution (960ng/ml)	0.5ml x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human DHA Antibody	1ml x1

- **Standard Preparation**

A120µL of the standard (960ng/L) was reconstituted with 120µl of standard diluent to generate a 480ng/L standard stock solution. The standard was allowed to be set for 15 minutes with gentle agitation before making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (480ng/L) in a ratio of 1:2 with standard diluent to produce 240ng/L, 120ng/L, 60ng/L, and 30ng/L solutions (Figure 2-6). Standard diluent serves as the zero standard (0 ng/L). Table 2-5 shows the dilution of standard solutions.

Table (2-5) Dilution of standard DHA

480ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
240ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent

120ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
60ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
30ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

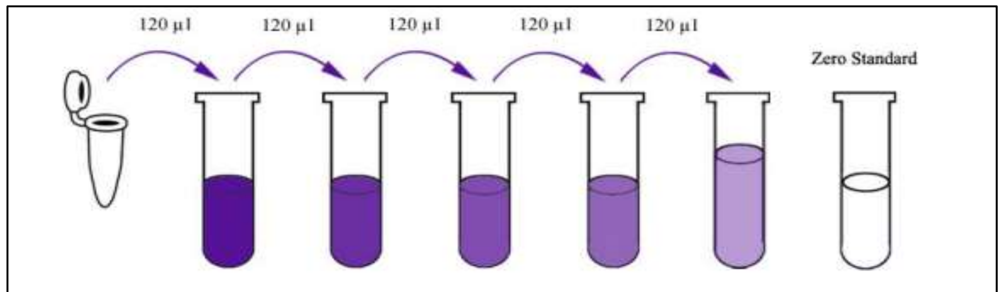

Figure (2-6) Dilution of Standard DHA

- **Procedure**

1. A 50µL of the standard was added to the standard well with the noted don't add a biotinylated antibody to the standard well because the standard solution contains a biotinylated antibody.
2. A 40µL of the sample was added to sample wells and then 10µL of anti-DHA antibody was added to sample wells and 50µL of streptavidin-HRP was added to sample wells and standard wells (Not blank control well). The plate was covered with a sealer and incubated for 60 minutes at 37°C.
3. The sealer was removed and washed the plate 5 times with wash buffer. Wells were automated washing, aspirated or decanted was done for each well and washed 5 times with wash buffer. The plate was blotted onto paper towels.
4. The substrate solution A (50µL) was added to each well and then 50µL substrate solution B was added. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in a dark place.

5. The stop Solution (50µL) was added to each well, and the blue color changed to yellow color immediately.
6. Each well's absorbance was immediately determined using a microplate reader.

- **Calculation**

The DHA concentration was calculated using a standard curve. A standard curve was constructed by plotting the absorbance for each standard on the y-axis against the concentration on the x-axis. (Figure 2-7).

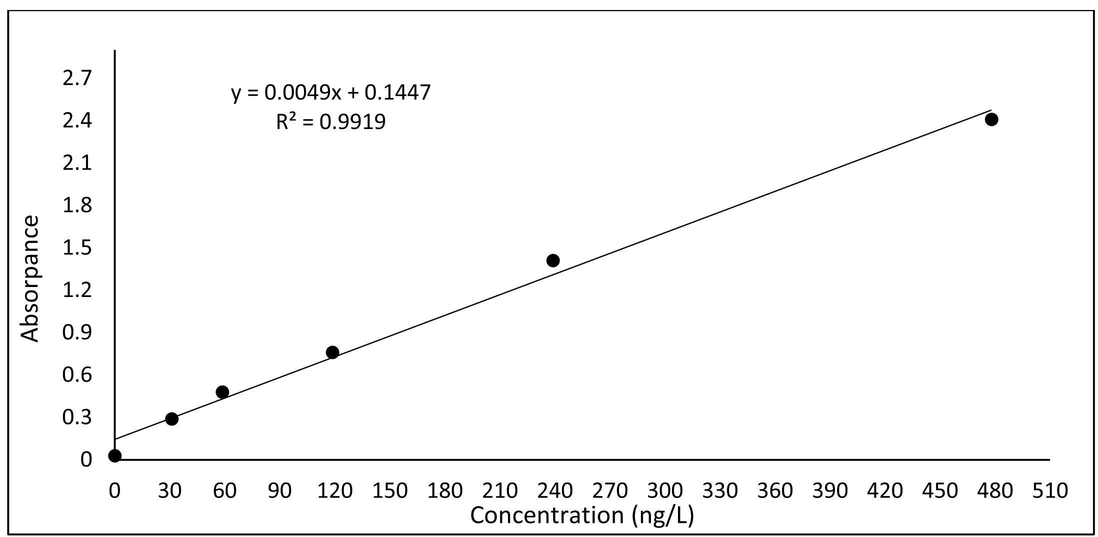

Figure (2-7): Standard Curve of Docosahexaenoic Acid

2.7.5. Determination of Homocysteine

- **Principle**

The assay principle of homocysteine is the same as the principle of sphingomyelin. It was mentioned above in the paragraph 2.7.2.

- **Reagents**

Components	Quantity (96T)
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human Hcy Antibody	1ml x1

- **Standard Preparation**

A120µL of the standard (64nmol/mL) was reconstituted with 120µL of standard diluent to generate a 32nmol/ml standard stock solution. The standard was allowed to be set for 15 minutes with gentle agitation before making dilutions. Standard points were prepared by serially diluting the standard stock solution (32nmol/ml) in a ratio of 1:2 with standard diluent to produce 16nmol/ml, 8nmol/ml, 4nmol/ml, and 2nmol/ml solutions (Figure 2-8). Standard diluent serves as the zero standard (0 nmol/ml). Table 2-6 shows the dilution of standard solutions.

Table (2-6) Dilution of Standard Homocysteine

32nmol/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent

16nmol/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
8nmol/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
4nmol/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
2nmol/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

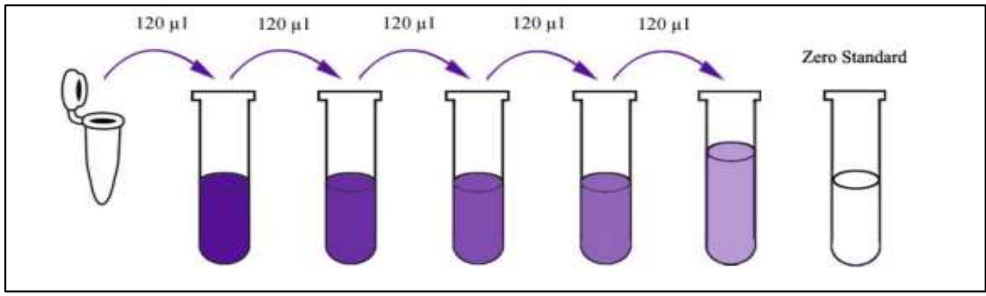

Figure (2-8) Dilution of Standard Homocysteine

- **Procedure**

1. A 50µL of the standard was added to the standard well with a note not to add a biotinylated antibody to the standard well because the standard solution contains a biotinylated antibody.
2. The sample (40µL) was added to sample wells and then 10µL anti-Hcy antibody was added to sample wells and 50µL streptavidin-HRP was added to sample wells and standard wells (Not blank control well). Well was mixed. The plate was covered with a sealer and then incubated for 60 minutes at 37°C.
3. The sealer was removed and washed the plate 5 times with wash buffer. Wells were automated washing, aspirated or decanted was done for each well and washed 5 times with wash buffer. The plate was blotted onto paper towels.
4. A 50µL of substrate solution A was added and then 50µL of substrate solution B was added to each well. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark.

5. Stop Solution (50µL) was added to each well, and the blue color changed to yellow color immediately.
6. Each well's absorbance was immediately determined using a microplate reader.

- **Calculation**

The homocysteine concentration was calculated using a standard curve. A standard curve was constructed by plotting the absorbance for each standard on the y-axis against the concentration on the x-axis (Figure 2-9).

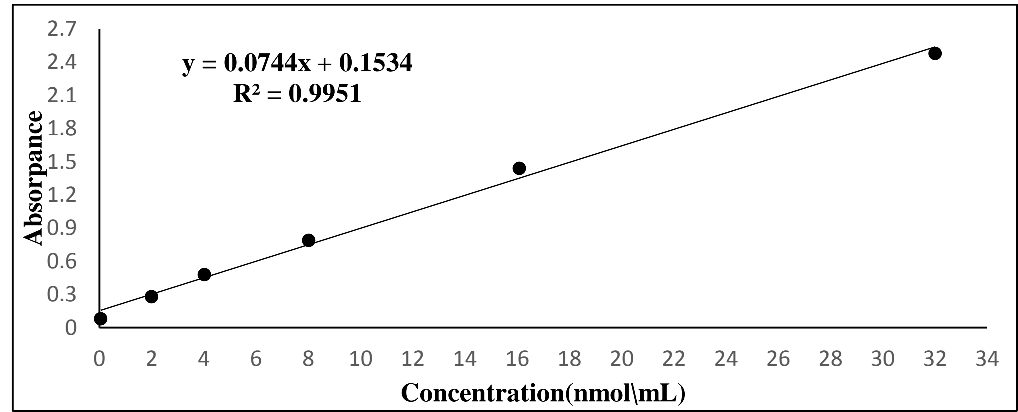

Figure (2-9): Standard Curve of Homocysteine

2.7.6. Determination of Folate

- **Principle**

The assay principle of folate same as the principle of sphingomyelin. It was mentioned above in the paragraph **2.7.2.**

- **Reagents**

Components	Quantity (96T)

Standard Solution (42ng/ml)	0.5ml x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human Folate Antibody	1ml x1

- **Standard Preparation**

A 120µL of the standard (24ng/mL) was reconstituted with 120µL of standard diluent to generate a 12ng/mL standard stock solution. The standard was allowed to be set for 15 minutes with gentle agitation before making dilutions. Standard points were prepared by serially diluting the standard stock solution (12ng/mL) in a ratio of 1:2 with standard diluent to produce 6ng/mL, 3ng/mL, 1.5ng/mL, and 0.75ng/mL solutions (Figure 2-10). Standard diluent serves as the zero standard (0 nmol/ml). Table 2-7 shows the dilution of standard solutions.

Table (2-7) Dilution of Standard of Folate

12ng/Ml	Standard No.5	120µL Original Standard + 120µL Standard Diluent
6ng/mL	Standard No.4	120µL Standard No.5 + 120µL Standard Diluent
3ng/mL	Standard No.3	120µL Standard No.4 + 120µL Standard Diluent
1.5ng/mL	Standard No.2	120µL Standard No.3 + 120µL Standard Diluent
0.75ng/mL	Standard No.1	120µL Standard No.2 + 120µL Standard Diluent

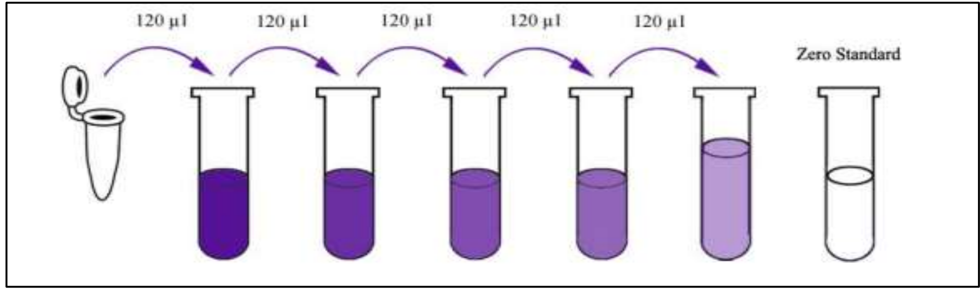

Figure (2-10) Dilution of Standard Folate

- ## Procedure

1. A 50µL of the standard was added to the standard well with don’t add a biotinylated antibody to the standard well because the standard solution contains a biotinylated antibody.
2. The sample (40µL) was added to sample wells and then 10µL of anti-folate antibody was added to sample wells and 50µL streptavidin-HRP was added to sample wells and standard wells (Not blank control well). Well was mixed. The plate was covered with a sealer and then incubated for 60 minutes at 37°C.
3. The sealer was removed and the plate was washed 5 times with wash buffer. Wells were soaked with 300µL wash buffer for 30 seconds to 1 minute for each wash. The plate was blotted onto filter paper.
4. The substrate solution A (50µL) was added to each well and then 50µL substrate solution B was added. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark.
5. Stop Solution (50µL) was added to each well, and the blue color changed into yellow immediately.

6. Each well's absorbance was immediately determined using a microplate reader.

- **Calculation**

A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis (Figure 2-11).

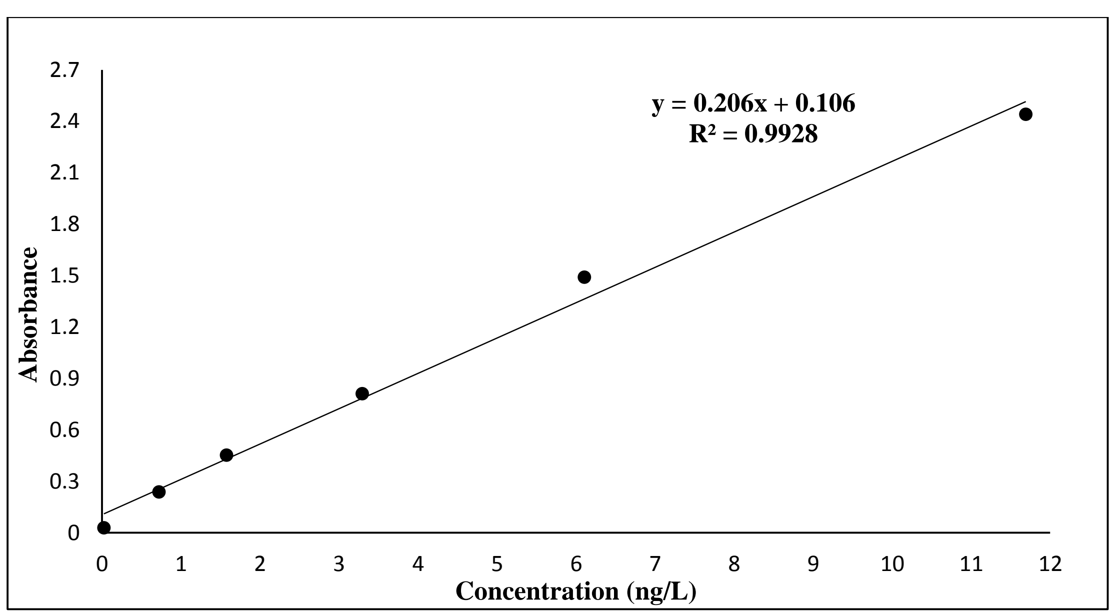

Figure (2-11): Standard Curve of Folate

2.7.7. Determination of Vitamin B_{12}

- **Principle**

Competitive Enzyme-Linked Immunosorbent Assay (ELISA) is used to determine vitamin B_{12} (Figure 2-12). Samples are added to the pre-coated plate. Then biotinylated antigen is added. The antigens in the samples compete with the biotinylated antigen to bind to the capture antibody and incubate. The unbound antigen is washed away during a washing step. An avidin-HRP is then

added and then incubated. Unbound avidin-HRP is washed away during a washing step. TMB Substrate is then added and color develops. The reaction is stopped by the addition of an acidic stop solution and the color changes into yellow which can be measured at 450 nm. The intensity of the color developed is inversely proportional to the concentration of vitamin B_{12} in the sample. The concentration of vitamin B_{12} in the sample is then determined by comparing the O.D. of the samples to the standard curve.

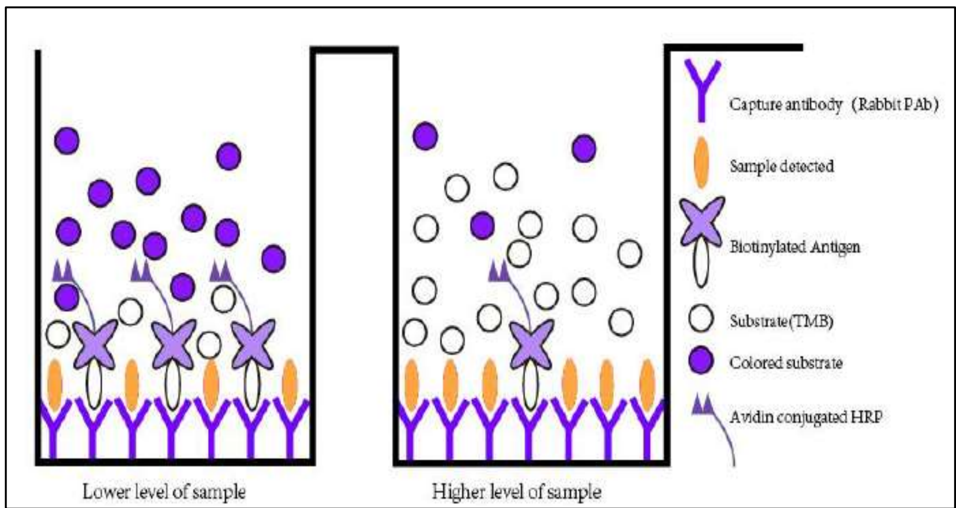

Figure (2-12): Competitive ELISA

- ## Reagents

Components	Quantity (96T)
Standard Diluent	3ml x1
Biotinylated Antigen, lyophilized	1 vial
Avidin-HRP Concentrate	100µl × 1 vial
Biotinylated Antigen Diluent	6ml × 1 vial
Avidin HRP Diluent	5.9ml × 1 vial
Substrate Solution A	6ml ×1 vial

Substrate Solution B	6ml ×1 vial
Stop Solution	6ml ×1 vial
Wash Buffer Concentrate (25x)	20ml × 1 vial

- ## Standard Preparation

A 150µL of the standard (24ng/ml) was reconstituted with 120µL of standard diluent to generate a 240ng/L standard stock solution which should be used within 24 hours. The standard was allowed to be set for 15 minutes with gentle agitation before making dilutions. Standard points were prepared by serially diluting the standard stock solution (12ng/ml) in a ratio of 1:2 with standard diluent to produce 120ng/L, 60ng/L, 30ng/L, 15ng/L, and 7.5ng/L solutions (Figure 2-13). Standard/Sample diluent only was added as the zero standards (0 ng/L). Table 2-8 shows the dilution of standard solutions.

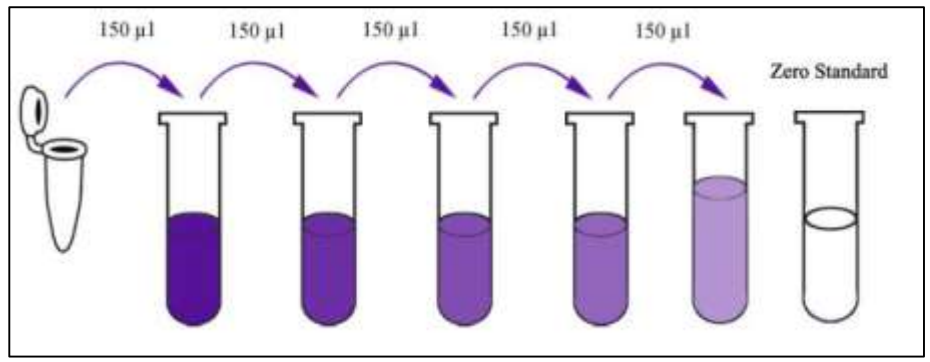

Figure (2-13) Dilution of Standard Vitamin B_{12}

Table (2-8) Dilution of Standard of Vitamin B_{12}

120ng/mL	Standard No.5	150µL Original Standard + 150µL Standard Diluent
60ng/mL	Standard No.4	150µL Standard No.5 + 150µL Standard Diluent
30ng/mL	Standard No.3	150µL Standard No.4 + 150µL Standard Diluent
15ng/mL	Standard No.2	150µL Standard No.3 + 150µL Standard Diluent
7.5ng/mL	Standard No.1	150µL Standard No.2 + 150µL Standard Diluent

- **Biotinylated Antigen Preparation**

A 1 mL of biotinylated antigen diluent was added to the biotinylated antigen vial. Then pipette all this solution back into the biotinylated antigen diluent vial to mix well and generate a 6 mL stock solution. The stock solution was allowed to sit for 10 minutes with gentle agitation before making dilutions.

- **Avidin-HRP Concentrate Preparation**

Briefly, a low-speed centrifuge of the avidin-HRP concentrates solution and then a pipette of all avidin-HRP into the Avidin HRP diluent vial well were mixed to generate a 6mL stock solution. The stock solution was allowed to sit for 10 minutes with gentle agitation before making dilutions.

- **Procedure**

1. Only substrate solution A, substrate solution B, and Stop solution were added into the blanck well as a blank control.

2. A 50 µL diluted standard was added to the standard well, 50µL of sample was added to the sample well, and 50 µL biotinylated antigen was added to each well. The plate was mixed and covered with a sealer after that plate was incubated for 60 minutes at 37C.
3. The sealer was removed and washed the plate 5 times with wash buffer. Wells were automated washing, aspirated or decanted was done for each well and washed 5 times with wash buffer. The plate was blotted onto paper towels.
4. A 50 µL of avidin-HRP was added to the standard well and sample well, then, the plate was covered with a sealer and incubated for 60 minutes at 37°C.
5. The sealer was removed and washed as described above.
6. Substrate solution A (50µL) and substrate solution B (50µL) were added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
7. The stop solution (50 µL) was added to each well, and the blue color changed into yellow immediately.
8. The absorbance was determined for each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

- **Calculation**

Vitamin B_{12} was evaluated using a standard curve. A standard curve was constructed by plotting the absorbance for each standard on the y-axis against the concentration on the x-axis (Figure 2-14).

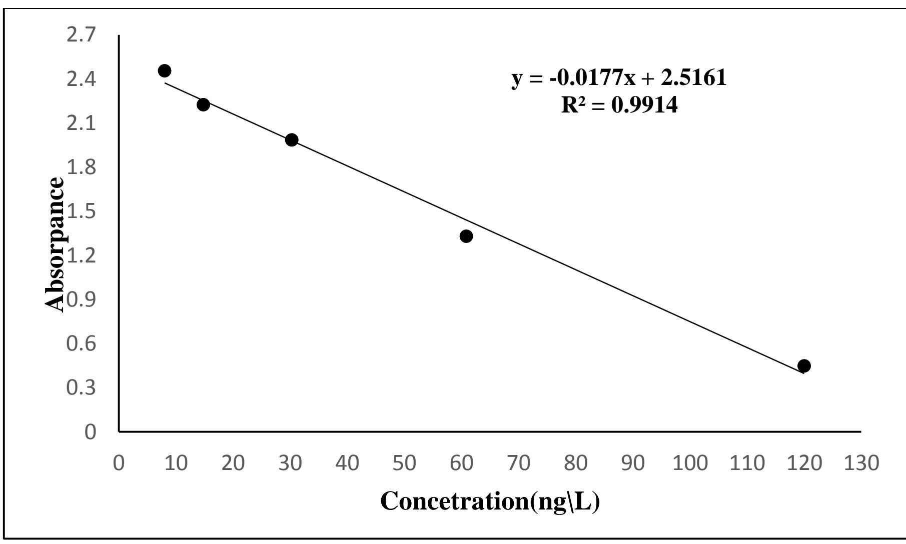

Figure (2-14): Standard Curve of Vitamin B_{12}

2.7.8. Determination of Trace Elements

The elements were determined using the Furnace Atomic Absorption Spectrometry (AAS) technique.

- **Principle**

The graphite furnace atomic absorption spectrophotometer technique (GFAAS) was used to determine trace elements in environmental and biological samples by conversion of an element into afree atomic state by means other than flame, such as using electronic furnaces and then measuring the absorbed light of a certain wavelength in the ground state. GFAAS is one of the most important of the five techniques of atomic absorption spectrometry which has a higher sensitivity that can be reached to the low detection limits (in ppb unit).
Trace elements such as (aluminum, zinc, and selenium) are determined in this way by using (SHIMADZU AA-6300 \ Japan) located in the laboratories of the College of Medicine, University of Kerbala as shown in Figure (2-15)

Figure (2-15) Atomic Absorption Spectrometry with Burner Head (SHIMADZU AA-6300)

2.7.8.1. Determination of Serum Selenium, Aluminum, and Zinc

Four standard solutions (2.5, 5, 7.5, 10) µg\L of the elements were prepared which were used for drawing the calibration curve as shown in Figures (2- 16), (2-17), and (2-18) respectively.

- **Procedures**

A small amount of samples of 20 µL was injected into a small graphite tube, which can then be heated by a wide range of temperatures to vaporize and atomize the analyst. The concentration of elements in samples was measured directly and continuously beyond the measuring of standard solutions depending on the calibration curve. The conditions for elements determination were listed in Tables (2-9), (2-10), and (2-11) respectively.

Table (2-9): Condition for Selenium Determination

Variable	Ideal condition
Atomizer	Graphite furnace
Fuel	Argon gas
Lamp current	10mA
Wavelength	196 nm
Slid width	0.7nm
Lighting mode	BGC-D2
Sample size	20μL

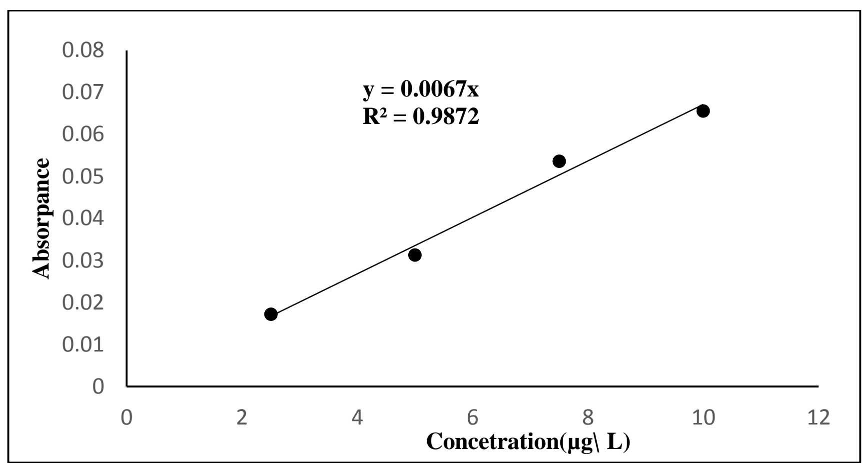

Figure (2-16): Standard Curve of Selenium

Table (2-10): Condition for Aluminum Determination

Variable	Ideal condition
Atomizer	Graphite furnace
Fuel	Argon gas
Lamp current	10mA
Wavelength	309.3nm
Slid width	0.7nm
Lighting mode	BGC-D2

Sample size	20µL

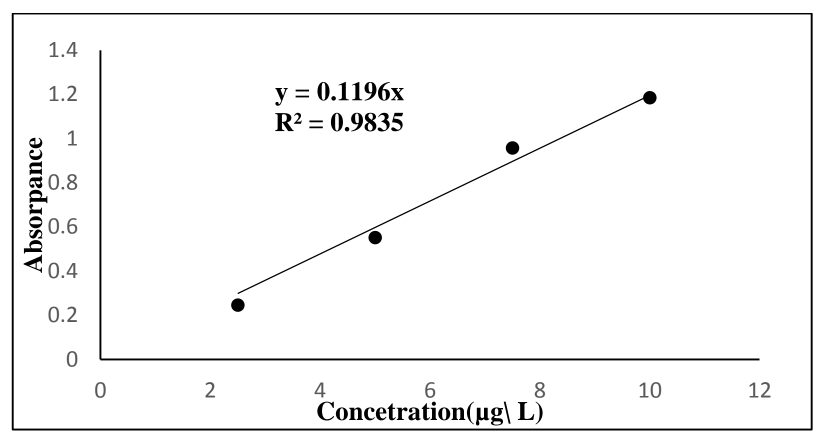

Figure (2-17): Standard Curve of Aluminum

Table (2-11): Condition for Zinc Determination

Variable	Ideal condition
Atomizer	Graphite furnace
fuel	Argon gas
Lamp current	10mA
Wavelength	213.9 nm
Slid width	0.7nm
Lighting mode	BGC-D2
Sample size	20µL

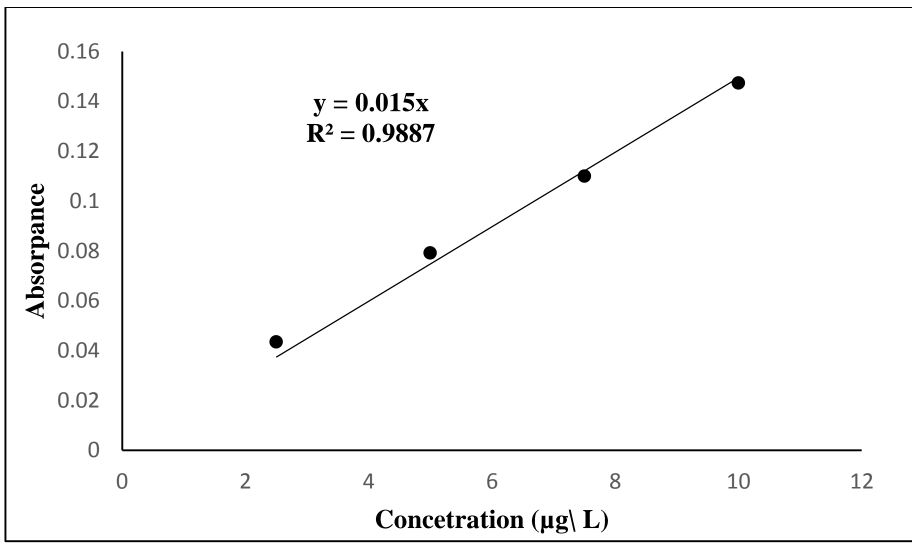

Figure (2-18): Standard Curve of Zinc

2.8. Statistical Analysis

The data results were tested using version 26 of the Statistical Package for the Social Sciences (SPSS), developed by IBM. The Shapiro test, which is a normality test, was employed to assess the distribution of the samples. The Shapiro test revealed a deviation from normal distribution, thus necessitating the utilization of the Mann-Whitney U test to analyze the data gathered in this study. The receiver operating characteristic (ROC) analysis test was employed to compute the area under the curve, which is widely regarded as a reliable measure of sensitivity and specificity in detecting and identifying. on the other hand, a linear regression model was employed to examine the association between parameters under study and autism spectrum disorder (ASD). To examine the relationship between the variables in this study, a correlation analysis was conducted. The statistical analysis in this study utilized Spearman's rank correlation coefficient (r). A p-value of 0.05 or below was deemed to be statistically significant in all conducted statistical tests.

Chapter Three

Results

and

Discussion

3. Results and Discussion

3.1. Characteristic Features:

The questionnaire was achieved by the parents of the children and psychological trainers employed at the autism center who oversee the care of children diagnosed with autism. Table (3-1) shows the clinical characteristics of ASD children. The results showed a higher percentage of male children with ASD than females, and most children with ASD who participated in this study had hyperactivity, and a large percentage of them lived in cities. Additionally, the academic achievement of most parents' children participants in this study from college grads and have job work. For assessing the hereditary factor, the results revealed that most of the participants had no hereditary ASD factor.

Autism is more prevalent in males than in females. Fombonne (2003) conducted a comprehensive analysis of the scientific research on autism epidemiology. It was observed that the sex ratios of autism, expressed as the ratio of males to females, varied between 2:1 and 16:1[70]. Dworzynski et al. (2012) discuss the preponderance of males to females, which is 4:1 in the general population. However, this ratio lowers to 10:1 for intellectually able girls and climbs to 2:1 for females with learning challenges (71).

According to the scientific literature, 50 to 70% of individuals with autism spectrum disorder (ASD) also present with comorbid attention deficit hyperactivity disorder (ADHD) [207]. Additionally, a study which was published in the Journal of Autism and Developmental Disorders in 2014 found that children living in urban areas were 1.5 times more likely to be diagnosed with autism than those living in rural areas [208].

Table (3-1): Clinical Characteristics of Children with ASD

Sample characteristics	ASD Children N (%)	Healthy children N (%)
Sex: Male Female	 32(76.1) 10(23.8)	 35(72.9) 13(27)
Age (y): mean ± SD	6.35±2.37	6.30 ±2.4
ASD severity level: Medium Under medium	 19(45) 23(55)	-----
Hyperactivity Yes No	 29(69) 13(30.9)	-----
Living: Villages and countryside City	 10(23.8) 32(76.19)	 18(37.5) 30(62.5)
Lack of oxygen at birth	Non	
Talking: Speaking Non-speaking	 25(59.50) 17(40.50)	-----
Hereditary No Yes	 10(23.8) 32(76.2)	-----
Father's academic achievement: Collegiate Secondary Primary Reads and writes	 22(52.4) 8(19) 10(24) 2(4.6)	 25(52) 12(25) 8(16.6) 3(6.4)
Mother's academic achievement: Collegiate Secondary Primary Reads and writes	 23(62.16) 3(8.11) 9(24.32) 2(5.41)	 20(42) 7(14.5) 13(27) 8(16.5)
Father's job: Employee Earner	 22(59.5) 15(40.5)	 29(60) 19(40)
Mother's job Employee Housewife	 20(54.05) 17(45.9)	 17(35) 31(75)

3.2. Distribution of Samples:

It was found, following normality tests that were conducted to explain the distribution of samples of each aspect of the study, that there was an abnormal distribution, as is illustrated in Figure (3-1). In light of the results

of this test, the most suitable strategy for carrying out statistical procedures was selected.

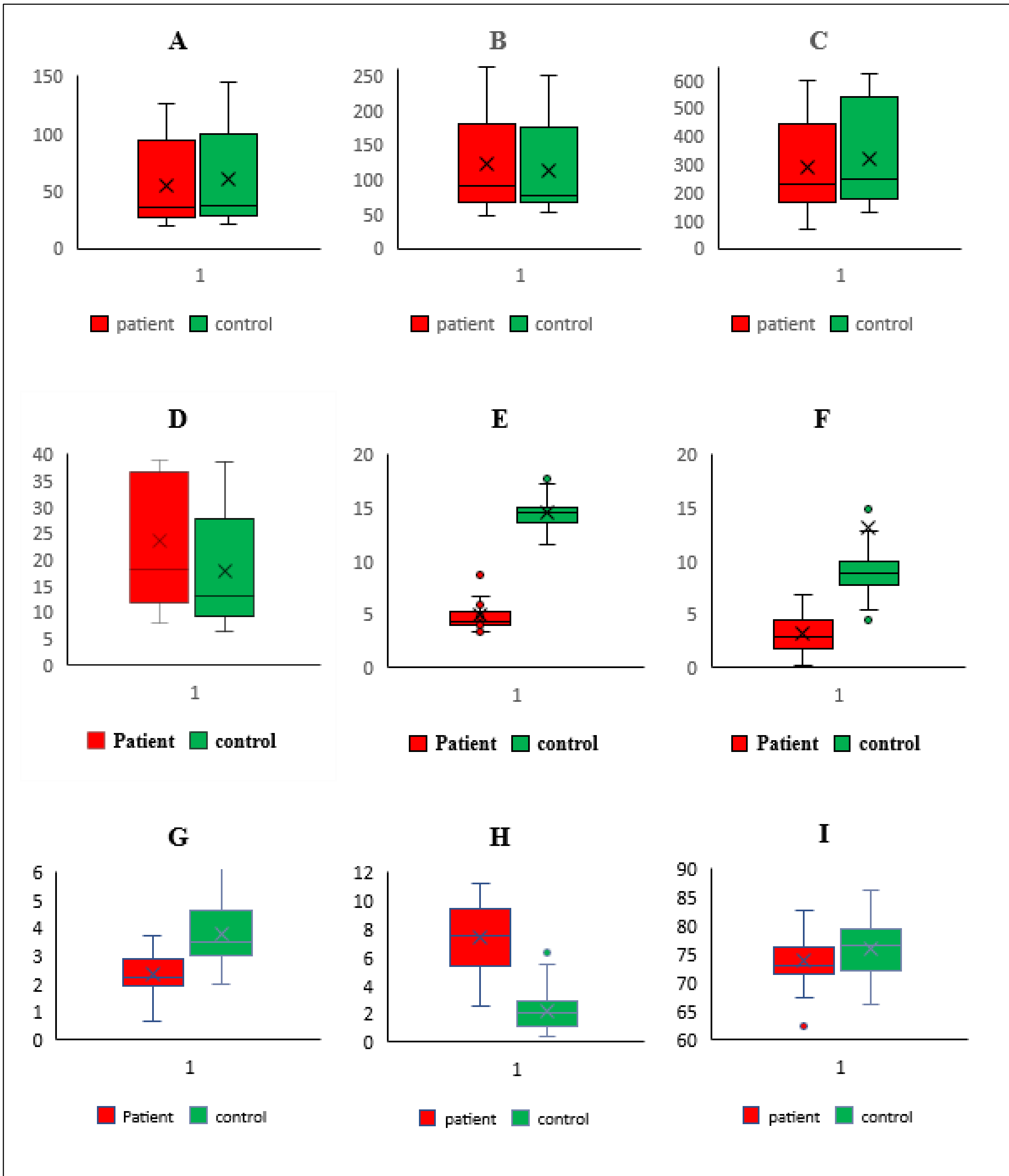

Figure (3-1) Box-whisker plots of serum (A)sphingomyelin (B) plasminogen and (C) docosahexaenoic acid (D) homocysteine (E) folate (F) vitamin B_{12} (G) selenium (H) aluminum (I) zinc levels in children with ASD and healthy group.

3.3. Determination of Sphingomyelin Plasminogen and Docosahexaenoic Acid

The three biomarkers were measured in the serum collected in ASD and control groups. In detail, the results presented in Table 3-2 show no insignificant increase in levels of sphingomyelin (OR:1.24, CI:0.517~2.970, *p*=0.456), plasminogen (OR:2.188, CI:0.893~5.356, *p*=0.384), and docosahexaenoic acid (OR:1.296, CI:0.540~3.109, *p*=0.619) when compared the children with ASD with a control group.

Table (3-2): The Concentration of Parameters Under Study between Children with ASD and the Healthy Group.

Parameters	Children with ASD N=42	Healthy Children N=48	O. R	CI (95%)	*P*- value
	Median (Range)	Median (Range)			
Sphingomyelin(mg\dL)	37.12(123.88)	36.15(106.84)	1.24	0.517~2.970	0.456
Plasminogen(ng/mL)	91.33(215.85)	76.425(196.77)	2.188	0.893~5.356	0.384
Docosahexaenoic acid (ng/L)	245.37(492.32)	227.88(531.38)	1.296	0.540~3.109	0.619

N: Number of samples.
ASD: Autism spectrum disorder
OR: Odd ratio.
CI: Confidence Interval 95%

The ASD children were classified into two subgroups according to Gilliam’s scale. The results were presented in Table 3-3 showed a significant increase in levels of sphingomyelin (OR:4.691, CI:1.289~17.068, *p*=0.014), plasminogen (OR:8.27, CI:2.024~33.77, *p*=0.001), and docosahexaenoic acid (OR:5.156, CI:1.412~18.831, *p*=0.001) when compared medium-intensity group with a control group. In contrast, the level of these biomarkers in under medium-intensity group, showed a significant decrease in levels of

sphingomyelin (OR:10.33, CI: 2.59-41.256, p=0.001), plasminogen (OR: 0.5, CI: 0.169~0.560, p=0.05), and docosahexaenoic acid (OR: 4.55, CI: 1.30-15.96, p=0.003).

Table (3-3): The Concentration of Sphingomyelin, Plasminogen, and DHA between the Subgroup for Children with ASD and the Healthy Group.

Parameters	Children with ASD						Healthy group (N=48)
	Medium-intensity cases (N=23)			Under medium-intensity cases (N=19)			
	Median (Range)	O. R	CI (95%)	Median (Range)	O. R	CI (95%)	Median (Range)
Sphingomyelin(mg\dL)	94.34(100.19)*	4.691	1.289-17.068	28.67 (53.76)*	10.33	2.59-41.256	37.12(123.88)
Plasminogen(ng/mL)	181.88(182.05)*	8.27	2.024-33.77	67.45 (47.37)*	0.5	0.169-0.560	76.42(196.77)
Docosahexaenoic acid (ng/L)	450.59(383.72)*	5.156	1.41218.831	174.24(220.98)*	4.55	1.30-15.96	245.37(492.32)

*P-value < 0.05, ASD vs. healthy group
N: Number of samples.
ASD: Autism spectrum disorder.
OR: Odd ratio.
CI: Confidence Interval 95%.

Biomarker variations across ASD subtypes have been evaluated by comparing two groups (Medium intensity and Under medium-intensity group). According to the findings, all the levels of the parameters in the medium-intensity group are significantly higher ($p<0.001$) than those in the low-intensity group as shown in Table 3-4.

Table (3-4): The Concentration of Sphingomyelin, Plasminogen, and DHA between Subgroups of Children with ASD

Parameters	Children with ASD		P-value
	Medium intensity cases (N=23)	Under Medium-intensity cases (N=19)	
	Median (Range)	Median (Range)	
Sphingomyelin(mg\dL)	94.34 (100.19)	28.67 (53.76)	< 0.001
Plasminogen(ng/mL)	181.88 (182.05)	67.45 (47.37)	< 0.001
Docosahexaenoic acid(ng/L)	450.59 (383.72)	174.24 (220.98)	< 0.001

ASD: Autism spectrum disorder
N: Number of samples.

Autism spectrum disorder (ASD) has been one of the most puzzling disorders of the previous decade. Sphingomyelin, Plasminogen, and Docosahexaenoic acid (DHA) are three important parameters that have received little attention.

Initiate, when comparing the ASD medium group with the control group, the results show that the ASD medium group has a higher concentration of Sphingomyelin, which may lead to hyperactivity in children. These findings are supported by a very interesting *in vivo* experiment in which a mouse model was used to evaluate the effect of different doses of propionic acid (PPA) can produce an increase in brain lipid profiles. After receiving the medication, these mice became extremely active [209].

Alterations in brain lipid profiles are thought to be a risk factor for autism spectrum disorder. Consequently, alterations in brain lipids have been linked to ASD. The brain's lipid profile can be altered by two different routes: an increase in dietary and enteric short-chain fatty acids. Therefore, using a rodent model, there may be a link between alterations or increases in brain sphingomyelin to the development of ASD-like behaviors. Additionally, ASD may be influenced by a change in the way the brain processes fatty acids [210]. The particular sphingomyelin found in brain lipids is crucial to the growth and maturation of the brain. Thus, any reduction may affect mental growth. Sphingomyelin concentration was low in the ASD under the medium group when compared to the control group. Because sphingomyelin supports brain myelination, a process strongly related to cognitive maturation and growth in the brain [211]. Decreasing sphingomyelin concentration has been shown to play a role in ASD development [212].

Plasminogen, via increasing the activity of tissue plasminogen activators, is a second important factor in the development of ASD. The results demonstrated that plasminogen levels are higher in children with ASD than in

the control group. Furthermore, Hasan Bozkurt (2021) found that any increase in blood Plasminogen in male ASD children will co-incidence with an increase in tissue Plasminogen activator (tPA) [213]. In other words, any elevation of blood Plasminogen led to an increase in tPA. Inside the blood vessel, the main function of tPA is a thrombolytic enzyme, and its main target is plasminogen. However, by contrast, in the brain parenchyma, tPA has been associated with multiple physiological and pathological events including synaptic plasticity and cell death [214]. Also, Tsirka *et al*. reported that tPA-deficient mice are resistant to neural degeneration [215]. In a recent study, tPA is involved in social behavior [216]. The results obtained support Bauman & Kemper's, 1994 hypothesis about the overgrowth in limbic structures, the overgrowth, and decreased growth of the cerebellum in males with ASD [217].

Omega-3 or N-3 fatty acids have various advantages, but they can also have negative side effects, such as enhanced lipid peroxidation [218]. Docosahexaenoic acid (DHA) is highly oxidized when it is found in high concentration, and the presence of its six double bonds in the 22-carbon fatty acyl chain increases its susceptibility to oxidation [219]. The quantity of pentadiene moieties contained in polyunsaturated fatty acids (PUFAs), on the other hand, is widely known to be strongly connected to the rate of oxidation. As a result, membranes enriched in DHA are more vulnerable to oxidative damage, and membrane oxidative changes have numerous potential repercussions in human pathophysiology. DHA levels were significantly higher in the ASD medium group than in the control group. DHA may fulfill a vital role in antioxidant pathways under certain conditions. Because DHA exhibits double bonds, three elements may mitigate oxidative stress by binding with it. Phosphatidylethanolamine plasmalogens (PE plasmalogens), diacyl-glycerophosphoethanolamine (diacyl-GPE), and phosphatidylcholine (PC) are the chemical structures. remarkably, any rise in DHA blood levels means an increase in the affinity of binding between those elements, and hence a

reduction in DHA's oxidation. DHA has already been shown to accumulate in overabundance and it has been proposed that DHA when bound to PC, is related to diminished cell growth and enhanced cytotoxicity [220].

Any fluctuations in plasma N-3 fatty acid levels, particularly DHA, have catastrophic consequences for the human brain. Furthermore, epidemiological data suggest that populations with lower levels of n-3 fatty acids, notably DHA, in plasma and brain tissue have a greater susceptibility to psychiatric disorders, such as depression and bipolar disorder [221,222]. In this study, the plasma level of DHA was low in ASD under medium group compared with the control group. In addition, children with hyperactivity disorder and adults with schizophrenia have been shown to have lower plasma phospholipid and DHA contents than the control [223].

3.4. Determination of Homocysteine, Folate and Vitamin B_{12}

The findings displayed in Table (3-5) indicate a statistically significant rise in the levels of homocysteine (OR: 2.00, CI: 0.792~5.05, *P*=0.02), as well as a highly significant decline in the concentrations of folate and vitamin B_{12} respectively [(OR:4.66, CI: 2.615~8.327, P<0.001), (OR: 9.25, CI: 3.66-23.339, p <0.001)] when comparing children with autism spectrum disorder (ASD) to a control group.

Table (3-5): The Concentration of Homocysteine, Folate, and Vitamin B_{12} between Children with ASD and the Healthy Group.

Parameters	Children with ASD (N=42) Median (Range)	Healthy group (N=48) Median (Range)	O. R	CI (95%)	*P*-value
Homocysteine(nmol\ml)	18.27(31.02)	13.134(31.47)	2.00	0.792~5.05	0.02
Folate(ng/l)	4.87(10.99)	14.430(16.51)	4.66	2.615~8.327	<0.001
Vitamin B_{12}(ng\L)	2.91 (13.69)	8.88 (190.04)	9.25	3.66-23.339	<0.001

N: Number of samples.
ASD: Autism spectrum disorder.
OR: Odd ratio.
CI: Confidence Interval 95%.

The findings presented in Table (3-6) showed a significant decrease in the concentration of folate and vitamin B_{12} (p-value less than 0.001) when comparing subgroups having different autistic intensity with the control group, while homocysteine concentration showed a significant increase only in medium-intensity cases of autism($p<0.001$) compared with the control group.

Table (3-6): The Concentration of Homocysteine, Folate, and Vitamin B_{12} between Subgroups for Children with ASD and Healthy group.

Parameters	Children with ASD						Healthy group (N=48)
	Medium-intensity cases (N=23)			Under medium-intensity cases (N=19)			
	Median (Range)	O. R	CI (95%)	Median (Range)	O. R	CI (95%)	Median (Range)
Homocysteine(nmol\ml)	36.75(3.71)**	0.348	0.199~0.609	12.265 (11.01)	1.190	0.4~3.544	13.134(31.47)
Folate(ng/l)	4.32 (5.89)**	0.063	0.01~0.42	4.915 (6.54)**	0.05	0.008~0.355	14.430(16.51)
Vitamin B_{12}(ng\L)	1.78 (2.78)**	0.075	0.01~0.44	4.315(4.89)**	0.33	0.196~0.568	8.88 (190.04)

*P-value < 0.05
**P-value<0.001
N: Number of samples
ASD: Autism spectrum disorder.
OR: Odd ratio.
CI: Confidence Interval 95%.

The Gilliam scale is utilized to assess the severity of autism spectrum disorder (ASD). It has been observed that children diagnosed with ASD can be categorized into two subgroups based on this scale: those exhibiting moderate intensity and those exhibiting mild intensity.

When comparing subgroups, it was observed that the concentration of homocysteine was significantly greater in the medium-intensity groups compared to the low-intensity group, whereas vitamin B_{12} levels were significantly lower ($p<0.001$) (Table 3-7).

Table (3-7): The Concentration of Homocysteine, Folate, and Vitamin B_{12} between Subgroups in Children with ASD.

Parameters	Children with ASD		*P*-value
	Medium-intensity cases (N=23)	Under Medium-intensity cases (N=19)	
	Median (Range)	Median (Range)	
Homocysteine(nmol\ml)	36.75 (3.71)	12.265 (11.01)	< 0.001
Folate(ng/l)	4.32 (5.89)	4.915 (6.54)	0.682
Vitamin B_{12}(ng\L)	1.78 (2.78)	4.315 (4.89)	< 0.001

ASD: Autism spectrum disorder
N: Number of samples.

Numerous epidemiological studies have shown a link between homocysteine, folate, and vitamin B_{12} and autism spectrum disorder (ASD), however, these studies lacked proper controls for confounding factors, a sizable sample size, and robust statistical analyses that would have affected how the data were interpreted. This study determined if the relationship between homocysteine, folate, and vitamin B_{12} levels and autism spectrum disorder (ASD) is linear or nonlinear. The results of this study support the evidence that increased homocysteine is associated with ASD linearly and may serve as a novel and sensitive biomarker for distinguishing children with ASD from healthy subjects. The finding that serum homocysteine was higher in children with ASD was robust after adjustment for relevant covariates, which was in line with a recently published meta-analysis that examined 31 articles involving 3304 subjects [224].

Few epidemiological researches have, to date, specifically examined the nature of the associations between homocysteine and ASD. The findings of this study showed a linear relationship between homocysteine and ASD, which could be partially accounted for by earlier *in vitro* research that demonstrated

increased homocysteine levels were neurotoxic in a dose-dependent way [225]. Emerging data points to mitochondrial dysfunction, methylation dysfunction, and increased oxidative stress as possible molecular pathways that may underlie these results and contribute to the pathophysiology of autism [226]. Additionally, it has been shown that high homocysteine levels have harmful effects on the neurological system and build up in animal brains, leading to aberrant brain energy metabolism, cognitive or neural malfunction, and behavioral changes [227,228].

On the other hand, the results of serum folate and vitamin B_{12} levels were lower in the children with ASD, one intriguing possibility is that cerebral folate deficiency under normal blood folate levels is caused by the impaired transport of folate across the blood-brain barrier [229,230], leads to homocysteine accumulation [231] Another possible reason is that homocysteine levels are strictly controlled by two metabolic pathways [232] one of them is which dysfunction in folate-independent re-methylation and transsulfuration pathways, but not in folate and vitamin B_{12}-dependent pathways, leads to elevated serum homocysteine concentrations. Human and animal experiments have reported an association between dysfunction in folate-independent pathways and increased homocysteine levels. One experiment in an animal model of ASD showed elevated serum homocysteine concentrations due to decreased betaine homocysteine methyltransferase expression, which is one of the folate-independent re-methylation-related enzymes [233]. An open-label trial in ASD children who were treated with folic acid and methylcobalamin showed that excess intake of folate and vitamin B_{12} did not significantly change homocysteine levels [234]. Other factors like *MTHFR* gene mutations can also induce elevated homocysteine without folate or B_{12} deficiency in children with ASD [235].

3.5. Determination of Trace Elements (Selenium, Aluminum, and Zinc)

The concentrations of three trace elements, namely selenium, aluminum, and zinc, were measured in the serum specimens of all participants. A comparison was then conducted between the two groups involved in the study (Table 3-8). Interestingly, a significant decrease in the levels of selenium and zinc elements while a significant increase in the levels of aluminum was observed in children with ASD. Specifically, the odds ratios (OR) and confidence intervals (CI) for selenium were found to be (OR: 5.25, CI: 1.96~14.08, p <0.001), for aluminum (OR: 7.68, CI: 2.64~ 22.34, p <0.001), and zinc (OR: 3.75, CI: 1.44~9.76, p =0.02).

Table (3-8): The Concentration of Selenium, Aluminum, and Zinc between Children with ASD and Healthy Control.

Parameters	Children with ASD (N=42) Median (Range)	Healthy group (N=48) Median (Range)	OR	CI (95%)	*P*-value
Selenium(µg\L)	2.26(3.11)	3.17(4.61)	5.25	1.96~14.08	< 0.001
Aluminum(µg\L)	7.57(8.61)	2.08(5.95)	7.68	2.64~ 22.34	< 0.001
Zinc(µg\L)	73.13(20.35)	76.55(20.11)	3.75	1.44~9.76	0.02

N: Number of samples.
ASD: Autism spectrum disorder.
OR: Odd ratio.
CI: Confidence Interval 95%.

The results presented in Table 3-9 showed a highly significant decrease in levels of selenium and aluminum (p<0.001) when compared between subgroups for children with ASD and healthy controls whereas, zinc levels show a significant decrease only in under medium intensity cases (p=0.04) compared to control group.

Table (3-9) The Concentration of Selenium, Aluminum, and Zinc between Subgroups for Children with ASD and Healthy Control.

Parameters	Children with ASD						Healthy group (N=48)
	Medium-intensity cases (N=23)			Under medium-intensity cases (N=19)			
	Median (Range)	O. R	CI (95%)	Median (Range)	O. R	CI (95%)	Median (Range)
Selenium(µg\L)	2.48(1.91) **	22.29	4.98~99.81	2.14(3.07) **	5.08	1.573~16.38	3.36(4.61)
Aluminum(µg\L)	8.34(8.61) **	0.01	0.00~0.03	7.56(6.48) **	0.6	0.389~0.712	3.42(4.61)
Zinc(µg\L)	73.00(16.62)	1.49	0.39~5.81	73.15(20.35) *	2.842	0.907~8.907	76.55(20.11)

ASD vs. healthy group
**P*-value < 0.05
***P*-value<0.001
N: Number of samples.
ASD: Autism spectrum disorder.
OR: Odd ratio.
CI: Confidence Interval 95%.

When comparing concentrations of trace elements between subgroups of children with ASD, it was observed that no significant difference between subgroups (Table 3-10).

Table (3-10) The concentration of Selenium, Aluminum, and Zinc between Subgroups for Children with ASD

Parameters	Children with ASD		*P*-value
	Medium-intensity cases (N=23)	Under medium-intensity cases (N=19)	
	Median (Range)	Median (Range)	
Selenium(µg\L)	2.48(1.91)	2.14(3.07)	0.13
Aluminum(µg\L)	8.34(8.61)	7.56(6.48)	0.76
Zinc(µg\L)	73.00(16.62)	73.15(20.35)	1.00

ASD: Autism spectrum disorder
N: Number of samples.

The results of this study suggest a notable correlation between reduced selenium levels and the occurrence of autistic spectrum disorder. Selenium, along with its related selenoproteins, plays a crucial role in various biological

processes, including the manufacture of thyroid hormones, DNA replication, reproductive capacity, and antioxidant defense [236]. Selenium (Se) plays a role in immune function by modulating the activity of activated T lymphocytes. Multiple studies have demonstrated that the administration of selenium (Se) supplements results in an accelerated and enhanced immune response [237]. The vital role of Se in brain function continues to be apparent. Selenoprotein P (SEPP1) is accountable for the transportation of selenium (Se) to the brain and serves a neuroprotective function in mitigating oxidative stress. Mice lacking the selenoprotein P1 *(SEPP1)* gene display evident impairments in brain function and also these mice had brain damage and eventual mortality as a result of insufficient dietary selenium [238]. The majority of selenoproteins have a role in the cellular response to oxidative stress [239], and the ability to counteract oxidative stress is crucial for proper neurodevelopment. The occurrence of heightened oxidative stress has been commonly documented in children diagnosed with ASD [240]. This oxidative stress is mostly attributed to the presence of reactive oxygen species (ROS) [241], elevated levels of lipid peroxidation [242], and diminished concentrations of antioxidants [243]. It is noteworthy that a particular antioxidant, glutathione peroxidase-1 (GPx1), which is a selenoprotein and recognized as a gene associated with ASD, regularly exhibits lower levels in children diagnosed with ASD [244]. In recent studies, it has been demonstrated that Se can inhibit a specific cellular mechanism known as ferroptosis, which is triggered by oxidative stress [245,246]. While the investigation of ferroptosis has predominantly focused on its occurrence in adult individuals with stroke, traumatic brain injury (TBI), and Parkinson's disease, there is a possibility that this mechanism also plays a role in nervous development. Therefore, Se deficiency may lead to heightened neuronal susceptibility to oxidative stress in children diagnosed with ASD. Even though Se is essential for healthy brain function, Se neurotoxicity has received a lot of attention lately [247]. Numerous studies have been conducted,

especially in the past few years, that indicate different inorganic and organic selenium compounds may have neurotoxic consequences if they are present at spur nutritional levels [248]. All things considered; everyone agrees that inorganic Se species selenite in particular are more neurotoxic than organic Se compounds [249].

The potential association between aluminum (Al) and ASD has been examined by multiple researchers [250,251]. Despite the prevalence of aluminum in the natural environment, it lacks any discernible biological role within the human body. Numerous studies have demonstrated that aluminum has the potential to induce developmental and immune impairments, disturb hormonal balance, exhibit neurotoxic properties, and impact cognitive functions and behavioral patterns. Aluminum (Al) is a neurotoxin that has been empirically proven to have detrimental effects on the human nervous system for several decades [252]. Previous studies have provided evidence indicating that individuals diagnosed with ASD exhibit a notable presence of Aluminum (Al) deposition inside the brain [253,254]. A study conducted by Melendez et.al demonstrated a correlation between elevated body aluminum levels and the presence of behavioral abnormalities in individuals with ASD [255]. Furthermore, empirical investigations have shown evidence of the capacity of Al compounds to negatively impact social behavior [256]. While there is currently no available evidence suggesting a direct link between aluminum (Al) exposure and catatonia, several researchers have presented findings that support the detrimental impact of Al on motor function [257,258].

The findings of this study revealed a statistically significant reduction in zinc levels in children diagnosed with ASD. The presence of zinc deficiency can have various impacts on the immunological and nervous systems of the human body. In both the developmental and adult stages of neurogenesis, Zn^{2+} is an essential component for several processes such as proliferation, migration,

differentiation, and survival. Neurons that are deficient in zinc exhibit reduced proliferation, differentiation, and apoptotic pathway activation [259]. In fact, data suggests that the hippocampal region is most likely the most vulnerable to a zinc deficit [260]. which reduces progenitor cell number and neuronal differentiation, thus causing irreversible impairment of learning and memory capacity during early development [261]. There is a suggestion that chronic zinc shortage may lead to reduced efficiency of the adaptive immune system and increased reliance on the innate immune system, even though the innate immune system also experiences damage in the presence of zinc deficiency [262]. According to a study conducted by Bonaventura et al. in 2015, it has been documented that zinc possesses anti-inflammatory properties. Additionally, a zinc deficiency has been found to elevate the levels of pro-inflammatory cytokines and enhance the activity of the central nervous system inflammasome [263]. Zinc has been found to protect against maternal insult induced by Lipopolysaccharide in animal models. This protection has been observed in the context of preventing aberrant behavior in object recognition tasks and abnormal sickness behavior following immune challenges. Furthermore, recent studies have shown that zinc can also prevent communication deficits in a mouse model of autism [264]. A recent review has also examined the relationship between zinc and the nervous system [265]. Considering the various functions of zinc, it seems advantageous to examine its classification into three overarching categories: structural roles, cellular signaling, and enzymatic co-factors. Zinc serves as an essential co-factor for many enzymes involved in DNA and RNA polymerization, histone modification, and DNA repair through the action of DNA ligase. Zinc plays a crucial role in various facets of protein synthesis inside the central nervous system (CNS) and functions as an autonomous factor in regulating gene expression [266]. In recent times, zinc has been recognized as a crucial constituent in structural proteins, particularly in the context of zinc-finger

motifs [257]. Proteins with widespread presence are frequently responsible for the composition of receptors found in the brain, such as estrogen, thyroid hormone, and glucocorticoid receptors [267]. Zinc plays a crucial role in facilitating the folding process and the subsequent development of the functional structure of these receptors [268]. Zinc has been associated with the development of olfactory, cerebellar, and hippocampus regions, and even a slight shortage of zinc has been demonstrated to impact memory and learning [269]. Previous studies have provided evidence indicating that temporary zinc deficit during gestation can have long-lasting effects on memory and learning abilities that extend into adulthood [270,271].

3.6. Correlation between Parameters Under Study

Spearman's correlation coefficient (r) test was used to examine the correlation between parameters measured in this study. Through results presented in Table 3-11, noted a positive strong correlation between sphingomyelin and plasminogen, and homocysteine, while a negative correlation between sphingomyelin and vitamin B_{12} $p<0.001$. Also, the results revealed a positive significant strong association between plasminogen and DHA and mid-correlation with homocysteine but plasminogen shows a negative correlation with VB_{12} ($p<0.001$). The findings demonstrate a statistically significant and robust correlation between DHA with homocysteine and VB_{12} $p<0.001$, $p=0.004$ respectively. Another link shown from these results is a negative correlation between selenium and aluminum ($p=0.027$).

Table (3-11): The Correlation between Parameters Under Study in Children with ASD

		Plasminogen	DHA	Homocysteine	Folate	VB_{12}	Selenium	Aluminum	Zinc
Sphingomyelin	**r**	0.897**	0.898**	0.695**	- 0.056	- 0.488**	- 0.090	- 0.065	0.030
	Sig.	< 0.001	< 0.001	< 0.001	0.758	0.004	0.618	0.720	0.867
Plasminogen	**r**		0.925**	0.552**	0.049	-0.423*	- 0.176	- 0.021	- 0.007
	Sig.		< 0.001	< 0.001	0.788	0.014	0.327	0.906	0.968
DHA	**r**			0.624**	- 0.067	- 0.488**	- 0.174	0.099	0.029
	Sig.			< 0.001	0.712	0.004	0.334	0.582	0.873

Homocysteine	**r**				- 0.121	- 0.704**	- 0.261	0.094	0.039
	Sig.				0.502	< 0.001	0.143	0.602	- 0.829
Folate	**r**					0.244	- 0.033	- 0.161	- 0.312
	Sig.					0.172	0.857	0.370	0.077
Vitamin B_{12}	**r**						0.287	- 0.242	- 0.140
	Sig.						0.105	0.175	0.437
Selenium	**r**							- 0.386*	- 0.078
	Sig.							0.027	0.664
Aluminum	**r**								0.226
	Sig.								0.206

3.7. Receiver Operating Characteristic Curve (ROC) for Parameters Under Study

3.7.1. Receiver Operating Characteristic Curve (ROC) for Sphingomyelin, Plasminogen and Docosahexaenoic Acid

The optimum cutoff points for serum Sphingomyelin, Plasminogen, and Docosahexaenoic acid levels were presented in Table (3-12) along with the area under curve (AUC) and sensitivity, and specificity (Figure 3-2). The value of the area under the curve for most parameters under study ranged between (0.7 to 0.8), also the percentage of sensitivity and specificity for most parameters ranged between 70% to 80% and this may be used this parameter as a biomarker to diagnose ASD in children after further study.

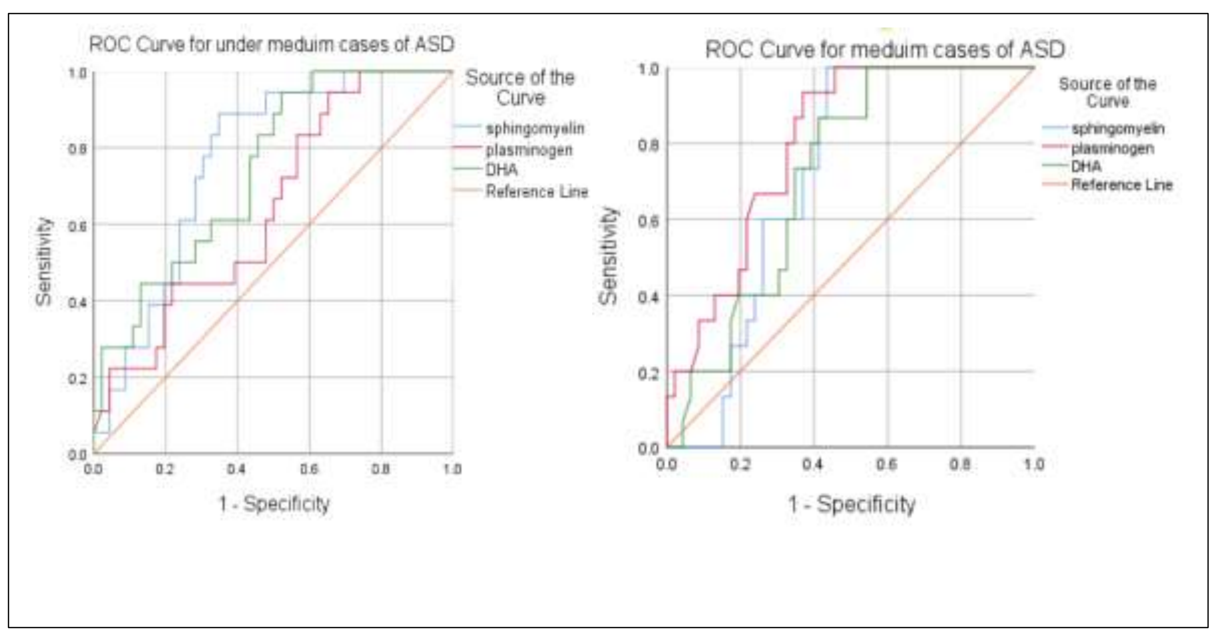

Figure (3-2) ROC Analyses of Sphingomyelin, Plasminogen, and DHA

Table (3-12): Coordinates of ROC Curve for Serum Sphingomyelin, Plasminogen, and DHA Discriminate ASD

Parameters / Variables	Sphingomyelin		Plasminogen		Docosahexaenoic acid	
	Medium cases of ASD (N=23)	Under Medium cases of ASD (N=19)	Medium case of ASD (N=23)	Under Medium cases of ASD (N=19)	Medium cases of ASD (N=23)	Under Medium cases of ASD (N=19)
The area under the curve	0.712	0.767	0.800	0.644	0.717	0.742
Cutoff value	>54.735	<32.31	>116.04	<76.80	>322.42	<206.7
Sensitivity%	73.3%	83.3%	80%	66.7%	73.3%	77.8%
Specificity%	63%	67.4%	68%	50%	65.2%	60%
Confidence Interval 95%	1.289 ~ 17.068	0.24 ~ 0.386	1.844 ~ 30.509	0.160 ~ 1.560	1.412 ~ 18.831	0.63 ~ 0.771
PPV%	40%	50	45	21	41	41
NPV %	87.88%	91	91.7	65.7	88.23	87
Accuracy	66	72	70	45	67	63

3.7.2. Receiver Operating Characteristic Curve (ROC) for Homocysteine, Folate and Vitamin B_{12}

The calculated area under the curve (AUC) for homocysteine was determined and it was 0.652. In contrast, the AUC values for vitamin B_{12} and folate exceeded 0.9, which is regarded as a good result (Figure 3-4). Table (3-13) displays the optimal cutoff values, sensitivity, and specificity for serum homocysteine, folate, and vitamin B_{12}. The sensitivity and specificity percentages for all parameters demonstrated favorable results, particularly in the case of folate and vitamin B_{12}, above 90%. Further investigation is required to utilize these biomarkers for the diagnosis of autism spectrum disorder (ASD) in pediatric populations.

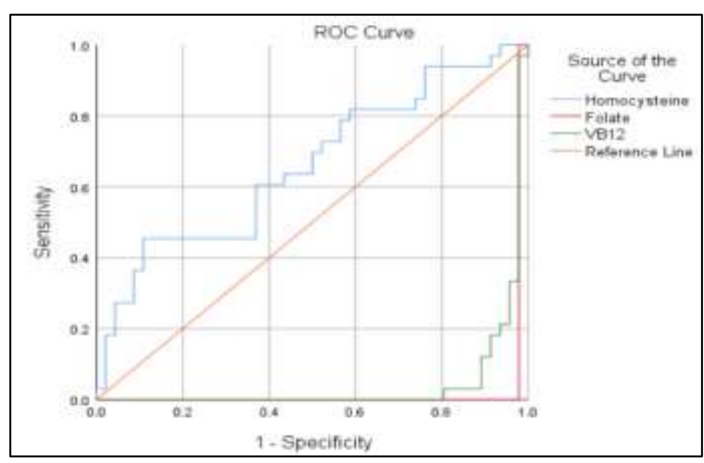

Figure (3-4) ROC Analyses for Homocysteine, Folate, and Vitamin B_{12}

Table (3-13): Coordinates of ROC Curve for Serum Homocysteine, Folate, and Vitamin B_{12} Discriminate ASD

Parameters / Variables	Homocysteine	Folate	Vitamin B_{12}
AUC	0.652	0.95	0.94
Cutoff value	>13.07	<12.085	<7.1
Sensitivity%	67%	95%	97.3%
Specificity%	50%	91%	80.4%
CI 95%	0.792~5.05	3.66~23.339	2.615~8.327
PPV%	66.66%	100%	100%
NPV %	50%	91%	80%
Accuracy	57%	95%	89%

AUC: Area under the curve.
PPV: Positive productive value.
NPV: Negative productive value.
CI: Confidence Interval 95%.

3.7.3. Receiver Operating Characteristic Curve for Selenium, Aluminum and Zinc

The area under the curve for all parameters was calculated by the ROC analysis test shown in Figure (3-6) and considered an excellent value [AUC of Se: 0.85, AUC of Al: 0.98, and AUC of Zn: 0.70]. The optimal cutoff values,

sensitivity, and specificity for serum trace elements are presented in Table 3-16. The percentage sensitivity and specificity for all trace elements under this study are considered very good pedigree and may be used as biomarkers to diagnose autism spectrum disorder after further study.

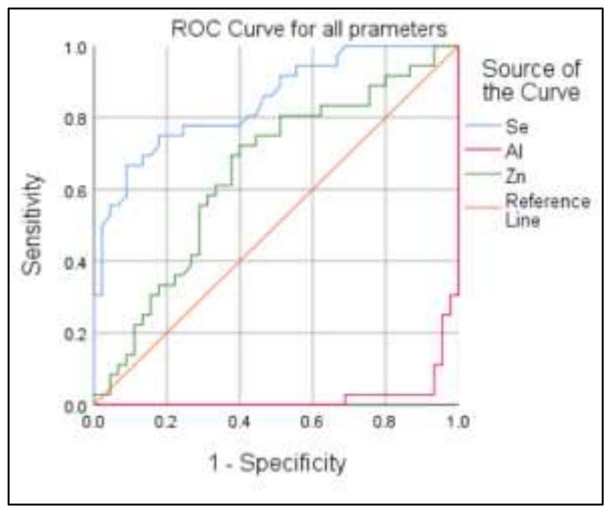

Figure (3-6) ROC Analyses of Trace Elements

Table (3-14): Coordinates of ROC Curve for Serum Trace Elements Discriminate

Parameters / Variables	Selenium	Aluminum	Zinc
AUC	0.85	0.98	0.70
Cutoff value	<3.12	>4.01	<76.94
Sensitivity%	87%	95%	78%
Specificity%	61%	85%	58%
CI 95%	0.792~5.05	3.66~23.339	2.615~8.327
PPV%	61%	85%	60%
NPV %	73%	94%	70%
Accuracy	70%	91%	65%

AUC: Area under the curve.
PPV: Positive productive value.
NPV: Negative productive value.
CI: Confidence Interval 95%.

3.8. Association Between Parameters Undre Study and Autism

The results presented in Table 3-15 indicate a strong positive association between the levels of homocysteine, selenium (Se), and zinc (Zn) and ASD (β: 0.43 CI: 0.004~0.083 *p*=0.03), (β: 0.48 CI: 0.280~0.679, *p* <0.001) and (β: 0.31 CI: 0.10~0.52, *p* =0.005) respectively whereas there is a negative association between their levels of vitamin B_{12}, folate, and Aluminum (Al) and ASD (β: -0.993 CI: -1.418~ -0.568 *p*<0.001), (β: -0.903 CI: -1.335~ -0.471 *p*<0.001), and (β: -0.83 CI: 0.71~0.95 *p* <0.001) respectively.

Table (3-15) Multiple Logistic Regression Analysis for the Associations between Parameters Under Study and Autism

Variables / Parameters	Regression Coefficient(β)	CI (95%)	*P*-value
Sphingomyelin	0.001	-0.002~0.004	0.5
Plasminogen	-0.001	-0.002~0.001	0.51
Docosahexaenoic acid	0.006	0.003~0.005	0.5
Homocysteine	0.43	0.004~0.083	0.03
Folate	-0.903	-1.335~0.471	<0.001
Vitamin B_{12}	-0.993	-1.418~0.568	<0.001
Selenium	0.48	0.280~0.679	<0.001
Aluminium	-0.83	0.71~0.95	<0.001
Zinc	0.31	0.1~ 0.52	0.005

Conclusions:

Conclusions

1. The biochemical markers in this study especially those that have a high sensitivity degree (sphingomyelin, plasminogen, DHA, folate, vitamin B_{12}, selenium, aluminum, and zinc) can be used to diagnose autism spectrum disorder in children.
2. Most biochemical markers in this study (sphingomyelin, DHA, folate, homocysteine, vitamin B_{12}, selenium, aluminum, and zinc) have high O.R. which they are considered a risk factor for children with ASD.
3. The biochemical markers in this study (homocysteine, folate, vitamin B_{12}, selenium, aluminum, and zinc) have a strong correlation positive or negative with ASD
4. Noted a strong correlation (positive or negative) between most parameters in this study.

Future Studies:

Future Studies

1. Conducting a study on children with a severe autism spectrum disorder.
2. Conducting studies on hair and nail specimens of autistic children and comparing them with serum specimens.
3. Studying the cupper element and determining the Zn/Cu ratio is important in diagnosing children with ASD.
4. Conducting a study on children with ASD using a large sample size.
5. Genetic study on children with ASD.

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Appendix A Research Questionnaire

List of informations
Sex: Male Female
Hyperactivity Yes No
Living: Villages and countryside City
Lack of oxygen at birth Yes No
Talking: Speaking Non-speaking
Hereditary No Yes
Father’s academic achievement: Collegiate Secondary Primary Reads and writes
Mother’s academic achievement: Collegiate Secondary Primary Reads and writes
Father’s job: Employee Earner
Mother’s job Employee Housewife

Appendix B Gilliam scale

جيليام-٢

مقياس جيليام التقديري لتشخيص التوحد – الاصدار الثاني

GILLIAM AUTISM RATING SCALE - SECOND EDITION (GARS-2)

نموذج ملخص الإستجابات

PRO-ED ،2005 ترجمة وتعريب: عبد الرحمن أحمد إعداد: جيمس جيليام

القسم الأول: معلومات تعريفية

إسم المفحوص: ________________

	اليوم	الشهر	السنة
تاريخ الاختبار	____	____	____
تاريخ الميلاد	____	____	____
العمر	____	____	____

الجنس: ☐ ذكر ☐ أنثى الصف الدراسي: ________

المدرسة: ________________

إسم المُقدر: ________________

إسم الفاحص: ________________

وظيفة الفاحص: ________________

القسم الثاني: ملخص الدرجات

المقاييس الفرعية	الدرجة الخام	الدرجة المعيارية	الرتبة	خمع
السلوكيات النمطية	____	____	____	١
التواصل	____	____	____	١
التفاعل الاجتماعي	____	____	____	١
مجموع الدرجات المعيارية		____		
مؤشر التوحد		☐	☐	٤

القسم الثالث: دليل التفسير

الدرجات المعيارية للأختبارات الفرعية	مؤشر التوحد	إحتمالية التوحد
٧ أو أكثر	٨٥ أو أكثر	محتمل جداً
من ٤ - ٦	٧٠ إلى ٨٤	من الممكن
من ١ - ٣	٦٩ أو أقل	غير محتمل

القسم الرابع: بروفايل الدرجات

الدرجة المعيارية	السلوكيات النمطية	التواصل	التفاعل الاجتماعي	مؤشر التوحد
	المقاييس الفرعية			
٢٠	•	•	•	١٥٠
١٩	•	•	•	١٤٥
١٨	•	•	•	١٤٠
١٧	•	•	•	١٣٥
١٦	•	•	•	١٣٠
١٥	•	•	•	١٢٥
١٤	•	•	•	١٢٠
١٣	•	•	•	١١٥
١٢	•	•	•	١١٠
١١	•	•	•	١٠٥
١٠	•	•	•	١٠٠
٩	•	•	•	٩٥
٨	•	•	•	٩٠
٧	•	•	•	٨٥
٦	•	•	•	٨٠
٥	•	•	•	٧٥
٤	•	•	•	٧٠
٣	•	•	•	٦٥
٢	•	•	•	٦٠
١	•	•	•	٥٥

القسم الخامس:

المقياس الفرعي الأول: السلوكيات النمطية

قدر كل بند من البنود الأتية طبقا لمعدل حدوثه، مستخدماً الخطوط الإرشادية الأتية لتحقيق أفضل مستوى للتقدير:

(صفر)لا يلاحظ إطلاقا لم تلاحظ إطلاقا أن هذا الشخص تصرف بهذه الطريقة.

(١) يُلاحظ بصورة نادرة: يتصرف هذا الشخص بهذه الطريقة مرة أو مرتين كل ست ساعات.

(٢) يُلاحظ أحيانا: يتصرف هذا الشخص بهذه الطريقة ٣-٤ مرات كل ست ساعات.

(٣) يلاحظ بصورة متكررة: يتصرف هذا الشخص بهذه الطريقة ٥-٦ مرات كل ست ساعات.

ضــــع دائرة حول الرقم الذي يصـــف ملاحظاتك لتصـــرفات الحالة في الظروف الطبيعة (مثلا: في معظم الأماكن، مع الأشـخاص المألوفين له، في الأنشـطة اليومية (العادية)، لا تنسـى الإجابة على كل البنود، وإذا كنت غير واثق من إجابتك على بند معين أجل إجابتك ولاحظ هذا الشـخص لمدة سـت سـاعات حتى تحدد تقديرك للبنود المؤجلة، تذكر كل فقرة يجب أن تحصل على تقدير.

		لا يلاحظ	يلاحظ نادرا	يلاحظ أحيانا	يلاحظ دائما
١	يتجنب دوام التقاء الأعين: ينظر بعيداً عندما يحاول احد أن ينظر إليه.	٠	١	٢	٣
٢	يحدق في الأيدي، الأشــياء والمواد الموجودة في البيئة لفترة لا تقل عن خمس ثوان.	٠	١	٢	٣
٣	ينقر بسرعه بأصابعه أو بيديه أمام عينيه لفترات مدتها خمس ثوان أو أكثر.	٠	١	٢	٣
٤	يأكل طعام معين ويرفض أن يأكل ما يأكله أغلب الناس – عادة.	٠	١	٢	٣
٥	يلعق، يتذوق، أو يحاول أكل أشــياء لا تُأكل (مثل : يد شــخص، ألعاب، كتب).	٠	١	٢	٣
٦	يشم أو يتشمم أشياء (مثل :ألعاب، يد شخص، شعر).	٠	١	٢	٣
٧	يدور أو يتحرك في دوائر.	٠	١	٢	٣
٨	يدير أشياء غير مصممة للتدوير (مثل :أطباق الفناجين، الفناجين، اكواب).	٠	١	٢	٣
٩	يهتز للأمام وللخلف أثناء الجلوس أو الوقوف.	٠	١	٢	٣
١٠	يقوم بحركات خاطفة، مندفعة، وسريعة عندما ينتقل من مكان لأخر.	٠	١	٢	٣
١١	يتبختر في مشيته (يمشي علي أطراف أصابعه).	٠	١	٢	٣
١٢	يخفق بيديه أو أصابعه أمام وجهة أو علي جنبيه.	٠	١	٢	٣
١٣	يصدر أصوات حادة (مثل :أي أي أي) أو أصوات أخري شبيهه كدافع أو حافز نفسي له.	٠	١	٢	٣
١٤	يصفع، يضرب، أو يعض نفسه أو يحاول إيذاء نفسه بأي طريقة أخري.	٠	١	٢	٣
			___+	___+	___

الدرجة الخام الكلية للسلوكيات النمطية	

Appendix

القسم السادس: مقابلة الوالدين

هذا الجزء يجب ملؤه عن طريق الأباء أو أحد القائمين على رعاية الطفل ممن لهم اتصال ودعم مباشر له.
أجب عن كل سؤال بتسجيل أحد الإجابتين (نعم) أو (لا)، أجب على كل الأسئلة.

• تأخر في:

١- التفاعل الاجتماعي:

	خلال الثلاث سنوات الأولى للطفل:	نعم	لا
أ	هل يظهر الطفل أي رغبه أو يجهز نفسه لرفعه عندما يحاول أحد الأباء حمله؟		
ب	هل يبكي الطفل أو ينزعج عندما يترك بدون مراقبة في سريره أو روضة الأطفال؟		
ج	هل يبكي الطفل أو ينزعج عندما يحمل أو يرفع؟		
د	هل يبكي الطفل أو ينزعج عندما يسلم من شخص بالغ لأخر؟		
هـ	هل حاول الطفل الانضمام لأفراد الأسرة في الأنشطة الجماعية (مثل: مشاهدة التلفاز)؟		

٢- اللغة المستخدمة في التواصل الاجتماعي:

	خلال الثلاث سنوات الأولى للطفل:	نعم	لا
أ	هل أستخدم الطفل كلمات فردية بعمر ١٦ شهراً؟		
ب	هل أستخدم الطفل عبارات تواصلية ذات معنى في عمر الثانية؟		
ج	هل تطور الطفل بشكل طبيعي من حيث اللغة (المناغاة، الثرثرة، التحدث دون انقطاع)؟		
د	هل اتبع الطفل التوجيهات (يبدو أنه يفهم ما يجب فعله عندما يُطلب منه فعل شيء ما)؟		
هـ	هل يبدو أن الطفل يتمتع بسمع طبيعي؟		

• أداء غير طبيعي في:

١- التفاعل الاجتماعي:

	خلال الثلاث سنوات الأولى للطفل:	نعم	لا
أ	هل يبتسم الطفل للوالدين أو الأشقاء عندما يبتسمون له أو يلعب معهم؟		
ب	هل بكى الطفل عندما أقترب منه أشخاص غير مألوفين له خلال العام الأول؟		
ج	هل قلد الطفل شخص آخر قبل سن الثالثة (مثل تقليد الأصوات أو اللعب بالعرائس)؟		
د	هل يبدو الطفل مشارك ومستجيب للأخرين؟		
هـ	هل يفضل الطفل قضاء الوقت مع الأخرين؟		

٢- اللغة المستخدمة في التواصل الاجتماعي:

	خلال الثلاث سنوات الأولى للطفل:	نعم	لا
أ	هل يستجيب الطفل لأسمه عند نداءه (مثل أن يلتفت وينظر نحو الشخص المنادي)؟		
ب	هل يطلب الطفل الأشياء أو يستخدم الإشارات لتوصيل ما يريده؟		
ج	هل يتبع الطفل التعليمات البسيطة (مثل تعال هنا، إديني حضن، اعمل باي باي)؟		
د	هل يبدو الطفل أنه يفهم ما يجب فعله عندما يُطلب منه فعل شيء ما؟		
هـ	هل يفهم الطفل (يُظهر القلق على وجهه) عندما يحزن أو يبكي أحد الوالدين أو الأشقاء؟		

٣- اللعب التخيلي أو الرمزي:

	خلال الثلاث سنوات الأولى للطفل:	نعم	لا
أ	هل شارك الطفل في لعب بالتقليد (مثل اللعب بالعرائس، أو الأبطال الخارقون، أو مجسمات الحيوانات) بشكل مناسب؟		
ب	هل تظاهر الطفل بأنه شخص آخر (مثل بابا، ماما، أو بطل خارق)؟		
ج	هل تظاهر الطفل بأن شيئاً ما هو شيء آخر (مثل هل تظاهر الطفل بأن عصا ما هي حصان ووضعها بين رجليه وتظاهر بأنه يركب حصاناً)؟		
د	هل تظاهر الطفل بأن لديه صديق أو حيوان خيالي؟		
هـ	هل لعب الطفل بالدمى متظاهراً بأنهم أشخاص حقيقيون؟		

الغرض من القسم الرابع هو توثيق ما إذا كان الفرد يلبي مجموعة من معايير التشخيص من الدليل التشخيصي والإحصائي الرابع-المعدل. إذا حددت "لا" لأي سؤال في هذا القسم، فإن الفرد يفي بمعايير التأخير أو الأداء غير الطبيعي.

القسم السابع: أسئلة رئيسية

تم تصميم الأسئلة التالية لمساعدة الفاحص للوصول إلى نتيجة تشخيصية. يجب على الفاحص الاهتمام بهذه الأسئلة لأنها تساعد في تفسير نتائج جيليام -٢.

١ ١. ما السلوكيات التي يفعلها الفرد والتي تجعلك تعتقد أنه مصاب بالتوحد؟ صف هذه السلوكيات على وجه التحديد قدر المستطاع.

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٢ متى حدثت هذه السلوكيات لأول مرة؟ يجب أن تظهر قبل سن ٣٦ شهراً.

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٣ هل تظهر هذه السلوكيات في كل الأماكن؟ يجب أن يظهر الفرد هذه السلوكيات في جميع الأماكن وبتواجد جميع الأشخاص، وليس في أماكن محددة أو عند وجود أشخاص محددون.

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٤ هل يمكن أن تكون السلوكيات نتيجة إعاقة أخرى؟ هل تم استبعاد التشخيصات الأخرى؟ كيف؟

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٥ من قام بتقييم الفرد وما هي النتائج؟ هل تم تقييم الفرد من قبل أشخاص مؤهلون لإجراء التشخيص (مثل أخصائي نفسي، طبيب نفسي)؟

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٦ ما التقييمات الأخرى التي أجريت بجانب جيليام -٢؟ هل تم إجراء اختبارات فردية أخرى مثل (اختبار الذكاء، اختبار التحصيل الدراسي، اختبار اللغة)؟

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٧ هل تم ملاحظة وجود دلائل في المجالات الثلاثة لتعريف التوحد (السلوكيات النمطية، التواصل، التفاعل الاجتماعي)؟

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٨ ما هي المجالات التشخيصية الأكثر تأثراً؟ ما هي الأعراض؟

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٩ ما مدى حدة الأعراض؟ كيف تعيق الأعراض الأداء الطبيعي؟

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١٠ ما المعلومات الأخرى التي يجب جمعها؟ من يستطيع الإدلاء بتلك المعلومات؟

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١١ ما التقييمات الأخرى المتاحة لمزيد من المعلومات؟

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الخلاصه

التوحد هو حاله معقده تحددها اسباب عديده احداها هو التركيز المفرط للمواد الكيميائيه الضروريه لدى الاطفال . في هذه الدراسه تم تقيم بعض الموشرات البيوكيميائيه المحتمله في عينات مصل الدم للاطفال المصابين بطيف التوحد مثل السفنكومالين ، البلازمينوجين ، حامض الدوكسوهكسانوك ،الهوموسيستين ، حامض الفوليك ، فيتامين B_{12} والعناصر النزره على وجه التحديد الزنك والسلينيوم والالمنيوم . هدفت هذه الدراسه عن امكانيه أيجاد علاقه بين مستويات المؤشرات البيوكيميائيه وشده التوحد .تضمنت هذه الدراسه 100 مشارك تم استبعاد 10 منهم واصبح العدد 42 طفل يعانون من اضطراب طيف التوحد(تم تصنيفهم اعتمادا على شده التوحد لديهم باستخدام مقياس جيليام الى مجموعتين هما الحالات متوسطه الشده والحالات دون المتوسطه الشده) و48 طفل اصحاء مطابقين في الاعمار والجنس لاطفال التوحد .استخدمت في هذه الدراسه تقنيتين الاولى تقنيه طيف الامتصاص الذري لتحليل العناصر النزره في امصال الدم والثانيه تقنيه الاليزا لتقدير الموشرات الحيويه الاخرى .

اظهرت النتائج عن وجود زياده ملحوظه في مستوياتالسفنكومالين (OR:4.691, CI:1.289~17.068, p=0.014) ،البلازمينوجين (OR:7.5, CI:1.844~30.509, p=0.001) وحامض الدوكساهكسانوك (OR:5.156, CI:1.412~18.831, p=0.001)عند مقارنه حالات متوسطه الشده من اطفال التوحد مع الاطفال الاصحاء في المقابل كان هناك نقصان ملحوظ في مستويات السفنكومايلين ، البلازمينزجين وحامض الدوكساهكسانوك (OR:10.33, CI: 2.59-41.256, p=0.001) ، (OR: 0.5, CI: 0.169~0.560, p=0.05)، (OR: 4.55, CI: 1.30-15.96, p=0.003) على التوالي عند المقارنه بين الحالات دون المتوسطه الشده مع الاطفال الاصحاء ، كما لوحظ ارتفاع في تركيز الهوموسيستين (OR: 2.00, CI: 0.792-5.05, p=0.02) وانخفاض كبير في تركيز الفوليت، فيتامينB_{12} [(OR:4.66, CI: 2.615~8.327, p<0.001), (OR: 9.25, CI: 3.66-23.339, p <0.001)] بالتتابع عند مقارنه اطفال التوحد مع الاصحاء.اما بالنسبه للعناصر النزره وجدت هذه الدراسه نقصان في تركيز السلينيوم والزنك(OR: 3.75, CI: 1.44~9.76, p =0.02) (OR: 5.25, CI: 1.96~14.08, p<0.001)على التوالي وزياده في تركيز الالمنيوم (OR: 7.68, CI: 2.64~ 22.34, p <0.001).

المساحه تحت المنحنى (ROC) كانت لمعظم الموشرات البيوكيميائيه ممتازه بالنسبه للسفنكومايلين والبلازمينوجين وحامض الدوكساهكسانوك اكثر من 0.7 في الحالات متوسطه الشده اما الفوليت وفيتامين B_{12} والسلينيوم والالمنيوم تتراوح بين (0.85- 0.95) وان هذه القيم تتوافق مع مستويات العاليه للحساسيه والنوعيه لهذه الموشرات البيوكيميائيه .اشارت النتائج الى وجود علاقه ايجابيه قويه بين اضطراب طيف التوحد ومستويات الهومويستين السلينيوم والزنك

(β: 0.43 CI: 0.004~0.083 p=0.03), (β: 0.48 CI: 0.280~0.679, p <0.001)
(β: 0.31 CI: 0.10~0.52, p =0.005) على التوالي.

بينما كانت هناك علاقه سلبيه بين اضراب طيف التوحد ومستويات الفوليت ، فيتامين B_{12} والالمنيوم
(β: -0.993 CI: -1.418~ -0.568 p<0.001), (β: -0.903 CI: -1.335~ -0.471 p<0.001),
(β: 0.83 CI: 0.71~0.95 p <0.001)

نستنتج من هذه الدراسه ان الموشرات البيوكيميائيه تعتبر عامل خطوره على اطفال التوحد واقترح استخدامها لتشخيص التوحد عند الاطفال وخصوصا التي اظهرت نسبه عاليه من الحساسيه بعد اجراء دراسات اضافية .

جامعه كربلاء
كليه العلوم
قسم الكيمياء

تقييم بعض المؤشرات الكيموحيوية لدى اطفال اضطراب طيف التوحد

الرساله مقدمه الى مجلس كليه العلوم \ جامعه كربلاء كجزء من متطلبات نيل شهاده الماجستير في علوم الكيمياء

من قبل

علي فضيل حمود

بكلوريوس علوم كيمياء \ جامعه كربلاء(2008)

بأشراف

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دكتوراه الكيمياء الحيوية

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